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Article

What Does “ITS” Say About Hybridization in Lineages of *Sarsia* (Corynidae, Hydrozoa) from the White Sea?

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Abstract: Hydrozoans are widely known for their complex life cycles. The life cycle usually includes an asexual benthic polyp, which produces a sexual zooid (gonophore). Here, we performed an extensive analysis of 183 specimens of hydrozoan genus *Sarsia* from the White Sea and identified four types of gonophores. We also compared the type of gonophore with haplotypes of molecular markers COI and ITS. Analysis of COI sequences recovered that the studied specimens relate to the species *S. tubulosa*, *S. princeps* and *S. lovenii*, and that *S. lovenii* specimens divide into two COI-haplogroups. More intraspecies genetic diversity was revealed in the analysis of ITS sequences. *Sarsia tubulosa* specimens divide into two ITS-haplotypes, and presumably, hybrid forms between these lineages were found. For *S. lovenii*, we identified 14 ITS-haplotypes as a result of alleles separation. Intra-individual genetic polymorphism of ITS-region is most likely associated with intraspecific hybridization between different haplotypes. The diversity of morphotypes is associated with the genetic diversity of the specimens. Thus, we demonstrated that the morphologically variable species *S. lovenii* is represented in the White Sea by a network of intensively hybridizing haplotypes. Hybridization affects the morphology and maturation period of gonophores and presumably affects the processes of speciation.

Keywords: Hydrozoa; Corynidae; *Sarsia lovenii*; hybridization; ITS; White Sea

1. Introduction

Hybridization can be defined as reproduction between members of genetically distinct populations [1,2]. Hybridization may be the result of interactions involving a wide range of types and levels of genetic divergence between the parental forms [2]. Hybridization has been considered as one of the mechanisms that influences the process of speciation [2,3]. The hybrid can reproduce either with its parental lineages (backcrossing or introgression) or only with similar hybrids [3]. In both cases, hybridization can lead to the emergence of novel features as well as new species altogether. Reticulate evolution caused by hybridization has played an important role in diversification of the several anthozoan genera [4–7]. Much less is known about the importance of hybridization in Medusozoa taxa including Hydrozoa [8–13] and Scyphozoa [14].

The complex life cycle of Hydrozoa includes pelagic medusa and sessile polyp stages [15–18]. Free-swimming medusae detach from benthic polyps or colonies, grow and spawn gametes shortly after maturation. Ciliated larva, planula, develops from the fertilized egg, settles and undergoes metamorphosis into the new polyp. However, reduction of the medusa stage is a widespread evolutionary trend among Hydroidolina and occurred independently in many phylogenetic lineages

[19–21]. Reduced gonophores lose many features of the medusa and usually form gametes staying attached to the mother colony.

Recently, an unusual morpho-genetic polymorphism was found in the hydroid *S. lovenii* (Corynidae) [13]. According to traditional views, colonies of *S. lovenii* produce reduced medusae named medusoids [22]. Medusoids form gonads without breaking away from the mother colony and lack ocelli and tentacles. Recently it was demonstrated, that *S. lovenii* has two morphotypes of gonophores: some colonies produce free-swimming medusae, while others produce medusoids [13]. The studied morphotypes belong to different genetic haplogroups, but the genetic distances between these haplogroups are minimal and correspond to the level of intraspecific variability. The possibility of hybridization between these haplogroups has also been experimentally proven. The results obtained were interpreted as a case of incipient speciation [13]. However, little attention was paid to hybridization of different lineages *S. lovenii* in the sea.

The aim of our work is a detailed analysis of the morphogenetic diversity of hydrozoans *Sarsia* in the White Sea, including the search for natural hybridization of lineages of *S. lovenii* using region of internal transcribed spacers of the ribosomal operon (ITS1-5.8S-ITS2).

2. Materials and Methods

Sampling and experimental cultures

The material was collected near the Pertsov White Sea Biological Station (Lomonosov Moscow State University) (66°34'N, 33°08'E) in 2015–2021 (Figure 1, Table S1).

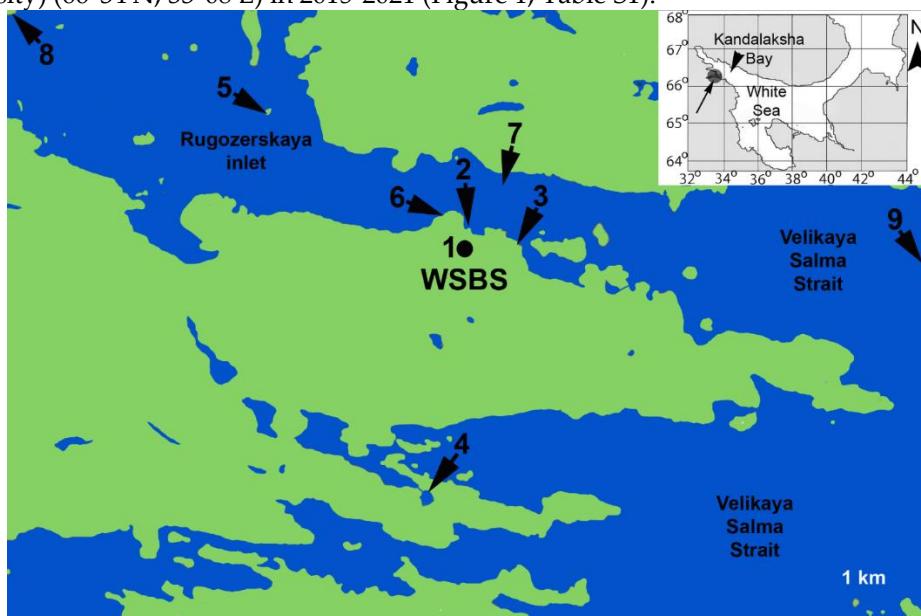


Figure 1. Sampling localities in the White Sea. The base geographic layer (USGS/NASA's Landsat 8 satellite data) was downloaded from the Landsat 8 satellite database (<http://libra.developmentseed.org>, accessed 29 May 2016). Landsat imagery courtesy of NASA Goddard Space Flight Center and U.S. Geological Survey. The base geographical layer of inset was generated using OpenStreetMap (© OpenStreetMap contributors, under the Open Database License. <https://www.openstreetmap.org/copyright/en>). Abbreviations: WSBS – White Sea Biological Station; 1 – Aquarium at WSBS; 2 – Pier of WSBS; 3 – Eremeevskie rapids; 4 – saline lake at the Green Cape; 5 – location "Luda"; 6 – location "Krest"; 7 – Rugozerkaya inlet, depth 5–15 m; 8 – Polovye islands; 9 – Velikaya Salma Strait, depth 40–60 m. .

Colonies were collected on different substrates near the shore. Medusae were collected manually from the surface of the water. Two hydroids were collected outside the White Sea: colony of *Sarsia lovenii* with medusoids was collected in the Barents Sea (Dalnezelenetskaya inlet) and medusa of *Sarsia* sp. was collected in the Bering Sea (Senyavin Strait). Medusae and colonies were photographed alive and fixed in ethanol (96%) for molecular phylogenetic analysis. Additionally, the collected

specimens were used for experimental cultures or for hybridization experiments. For experimental crossing, ready-to-spawn medusae and medusoids of *Sarsia* spp. were collected and maintained in small containers (200 ml) with filtered (0.2 microns) seawater at a temperature of 10-12° C. Females of *S. lovenii* with "medusoid" morphotype were placed together with male medusae of *S. lovenii*, and female medusae of *S. lovenii* were placed with males medusoids of *S. lovenii* (Table 1). In addition, *S. tubulosa* were crossed with *S. lovenii* medusoids.

Table 1. Schemes of crossing experiments and related DNA isolates.

Schemes of crossing	DNA isolates
<i>S. lovenii</i> medusa female x <i>S. lovenii</i> medusoid male	H140, H144, H151, H153, H157, H168
<i>S. lovenii</i> medusa male x <i>S. lovenii</i> medusoid female	H149, H150, H158, H159, H170
<i>S. lovenii</i> medusa female x <i>Sarsia</i> sp. male	H122
<i>S. lovenii</i> medusoid female x <i>S. tubulosa</i> male	H163
<i>S. tubulosa</i> female x <i>S. tubulosa</i> male	H249
<i>S. lovenii</i> medusa female x <i>S. lovenii</i> medusa male	H238, H251
<i>S. lovenii</i> hybrid F2: hybrid F1-males (H159+H140) x <i>S. lovenii</i> medusa female	H233, H236

New COI and ITS sequences for 140 specimens were obtained (Table S1). Previously obtained sequences were also used for analysis (the list of specimens see in [13]). In total, 183 specimens were used for analysis (Table 2): 143 specimens collected in the sea (including 42 medusa-specimens and 101 polyp-specimens), 40 specimens maintained in the laboratory (including 18 specimens obtained by experimental hybridization). For 99 specimens, we observed the mature gonophores (including medusa specimens) or experimentally induced their formation.

Table 2. Number of specimens of *Sarsia* spp. collected in different years.

Year of collection	Sampling location	Number of specimens	Number of medusa-specimens/ Number of polyp-specimens with mature gonophores
2015	Aquarium	6	0/1 [13]
2016	In the Sea, WSBS	9	4/0 [13]
2017	In the Sea, WSBS	23	22/1 [13]
2018	In the Sea, WSBS	5	1/4 [13]
2019	In the Sea, WSBS, Bering Sea, Barents Sea	43	1/10
2019	Aquarium	17	0/5
2019	Aquarium (crossing experiment)	13	0/11
2020	In the Sea, WSBS	43	14/8
2020	Aquarium (crossing experiment)	4	0/0
2021	In the Sea, WSBS	20	0/17

Morphological Analysis

To distinguish different morphotypes of gonophores in collected specimens and experimental colonies we analyzed: if gonophore detaches from the mother polyp as a free-swimming medusa or not; if edges of the bell with tentacles are bent inward or not, if bell unfolds before detachment or not; the presence of tentacles and ocelli on the tentacle bulbs; the presence of an incomplete nematocyst ring in epidermal part of bulbs; the shape of the bell and the size of the manubrium, the presence or absence of gonads at the gonophore before the detachment.

Molecular Analysis

DNA was isolated with a Diatom kit (Diatom DNA Prep 100 kit, Isogen Laboratory, Moscow, Russia) according to the manufacturer's protocol. The cytochrome c oxidase (COI) subunit fragment I and internal transcribed spacers of the ribosomal operon 18S-ITS1-5.8S-ITS2-28S rRNA (ITS) were amplified from isolated DNA with the following primers pairs: SR6R (AAGWAAAAGTCGTAACAAGG) and LR1 (GGTTGGTTCTTTCCCT) for 18S-ITS1-5.8S-ITS2-28S

rRNA [23, 24] with program 95 °C for 5 min; followed by 34 cycles of 15 s at 94 °C, 30 s at 52 °C and 60 s at 72 °C and then a final extension of 5 min at 72 °C; and SAR-F (TTGGGGCTTCGCCGGTAT) and SAR-R (CAGGATCACCTCCTCCTGC) for COI (*Sarsia*-specific primers, current study) with program 95 °C for 5 min; followed by 34 cycles of 15 s at 95 °C, 30 s at 50 °C and 60 s at 72 °C and then a final extension of 5 min at 72 °C. The polymerase chain reaction was carried out in a reaction volume of 20 µl, which included 4 µl of 5x Screen Mix solution (Evrogen, Moscow, Russia), 0.5 µl of each primer, 1 µl of DNA and 14 µl of sterile water. Amplification was also used in a volume of 25 ml, which included 5 ml of 5x Taq Red Buffer (Evrogen Lab), 0.5 ml of polymerase (HS-Taq Polymerase by Evrogen Lab), 0.5 ml of dNTP (50 µM stock), 0.3 ml of each primer (10 µM stock), 1 ml of DNA and 17.7 ml of sterile water (MilliQ). Sequencing was carried out at Evrogen (Russia) in an ABI Prism 3500 Genetic Analyzer (Applied Biosystems). Accession numbers of the sequences generated in the present study are listed in Table S1: accession numbers COI (OQ859724 – OQ859863), accession numbers ITS will be provided during review.

DNA Cloning

Specimen H122 is an experimental hybrid of female medusa *S. lovenii* and male medusa *Sarsia* sp. Specimen H144 is an experimental hybrid of female medusa *S. lovenii* and male medusoid *S. lovenii*. ITS of H122 and H144 specimens were isolated from the genome DNA samples with gene-specific SR6R and LR1 primers pair. Amplified fragments were cloned into the pAL-TA vector (Evrogen, Russia). Three clones have been sequenced from each plate.

Phylogenetic Analysis

Sequences were assembled and checked for improper base calling with CodonCode Aligner software (www.codoncode.com/aligner). Sequences were aligned using the MUSCLE [25] algorithm in MEGA 6 software [26]. The final alignments resulted in dataset comprising of 624 bp for the COI. JModelTest 2 [27] was used to estimate the best substitution model for each partition based on the Bayesian information criterion (BIC). The GTR+G model was found to be optimal for the COI dataset. Bayesian phylogenetic trees were built in PhyloBayes 3.3 [28]. The analysis was performed with random starting trees and 10 million generations. Two MCMC chains were run in parallel, and the analyses were stopped when the maximum discrepancy of bipartitions between chains was below 0.01. Final phylogenetic tree images were rendered in FigTree 1.4.0. Maximum Likelihood Phylogenetic analysis was performed in IQTree v.2.0-rc2 software [29] with standard algorithm. The best model of nucleotide substitution (GTR+F+G4) was chosen using ModelFinder [30] according to Bayesian information criterion (BIC). One thousand bootstrap replicates were generated for the analysis.

A haplotype network for COI dataset was constructed using the TCS network inference method [31] within PopART software (<http://popart.otago.ac.nz/index.shtml>). According to a constraint of the method, we used a reduced COI dataset without undefined states of nucleotides. Haplogroups of *S. lovenii* were identified in accordance to [13] or according to the morphology of the specimens.

For the analysis of ITS-sequences with heterozygotes we used Champuru v. 1.1 [32] (<https://eeg-ebe.github.io/Champuru/input.html>) to determine the haplotypes of heterozygous individuals without cloning, simply by analyzing the patterns of double peaks in the forward and reverse chromatograms (marked in results as phase-1 and phase-2). This method is well suited for separating haplotypes that differ only in one deletion locus, which is typical for different interlineage hybrids of *S. lovenii*. Sequences with one heterozygote we manually divided into two alleles (marked in results as allel-1 and allel-2). We trimmed the ends with unknown bases to align the length of all sequences. In addition, we excluded from analysis several short sequences less than 500 bp (H150, H157, H161, H170) and sequences with two single heterozygotes (H97, H118, H153, H184). ITS fragment of several *S. tubulosa* specimens, containing many single heterozygotes, also cannot be divided to haplotypes. The final alignments resulted in dataset comprising of 509 bp for the 214 sequences/ 152 specimens. We export dataset in RDF-format (Roehl Data File) using DnaSP 5.10 software with option «considered sites with gaps/missing» [33]. A haplotype network was constructed using the median

joining algorithm [34] within NETWORK 10.2.0.0 software (Fluxus Technology Ltd, www.fluxus-engineering.com).

3. Results

3.1. Morphotypes of Gonophores in Specimens of *Sarsia* spp

We identified four morphotypes of gonophores: free-swimming medusa (53 specimens), medusoid (17 specimens), attached medusa (17 specimens) and "abnormal medusoid" (12 specimens) (Figure 1, Figure S1, Table S1, [13]).

Morphotype I "medusoid" is characterized by the absence of tentacles and eyes on tentacle bulbs (Figure 2A). Tentacular bulbs are present, but they are significantly reduced compared to other morphotypes, they lack a C-shaped nematocyst zone. The bell is oval, noticeably narrowed in the distal part. The manubrium is encircled by gonads. Manubrium with gonads occupies a large part of the subumbrellar cavity. Gonads on the manubrium appear early in the development, sometimes they cover even the most distal part of the manubrium. There is no functional mouth, the medusoid does not feed. When medusoid is ripe, it is possible to observe a series of bell contractions, due to which gametes are expelled from the subumbrellar cavity. Colonies of *S. lovenii* with ripe medusoids were collected in the sea from June till July, and also medusoids were produced by some experimental colonies (Figure S1, Table S1).

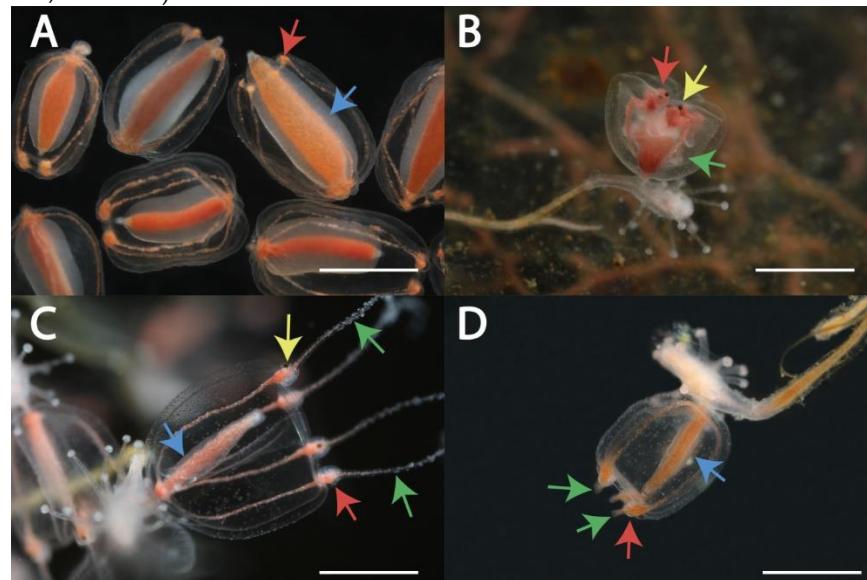


Figure 2. Four morphotypes of gonophores in specimens of *Sarsia lovenii* from the White Sea: morphotype I - medusoid specimen H347 (A), morphotype II - bud of free-swimming medusa specimen H167 (B), morphotype III - attached medusa specimen H140 (C), morphotype IV - "abnormal medusoid" specimen H341 (D). Medusoids (A) were detached from the polyps. Abbreviations: yellow arrows indicate ocelli, green arrows indicate tentacles, red arrows indicate tentacular bulbs with nematocyst belt (B-D) or without nematocyst belt (A), blue arrows indicate gonads covering manubrium. Scale bars 1 mm.

Morphotype II, medusa, detaches from the mother colony when it is completely developed. Further growth and maturation of gonads occurs in the feeding free-swimming medusa. Medusae of *Sarsia* spp. can be identified by morphological characters such as size of bell, morphology of tentacle bulbs and apical knob, position of gonads over manubrium [13,22]. We observed the development of medusa buds in *S. lovenii*. Late medusa buds are characterized by the presence of tentacles and eyes on tentacular bulbs, the edges of the bell are bent inward, and tentacles are inside the bell (Figure 2B). The medusa turns the tentacles out shortly before the detachment from the mother polyp, when the bell begins to contract. Medusae or medusa buds of *Sarsia* spp. were collected at the sea from March till July, and also obtained on some experimental colonies (Figure S1, Table S1).

Morphotype III, "attached medusa", is similar to a new-born free-swimming medusa by shape of the bell, however, gonophores usually do not detach from the mother colony (Figure 2C). Such attached medusae have relatively short tentacles and tentacle bulbs with ocellus. Late gonophores have a fully expanded bell, outward located tentacles, and are capable of periodic contraction. Mouth opening locates at the end of the manubrium. Gonads cover manubrium as a tube and can be observed in proximal and middle parts of manubrium. If mother polyp is resorbed, the gonophore may break away from the mother colony and swim near the bottom and even be able to feed. Morphotype "attached medusa" were formed on colonies that were obtained as a result of experimental crossing between the morphotype "medusa" and the morphotype "medusoid", both in the case of crossing individuals of *S. lovenii*, and in the case of interspecific crossing between the medusoid *S. lovenii* and the medusa *S. tubulosa*. Moreover, gonophores with such a morphotype were found in the sea on 12 May 2021 (Figure S1, Table S1).

Morphotype IV, "abnormal medusoid", differs from the typical medusoid by well-developed tentacular bulbs often elongated into short rod-shaped tentacles (Figure 2D). C-shaped zone of nematocysts is also visible at bulbs. The shape of the bell is the most similar to the morphotype "attached medusa". However, such gonophores lack ocelli on tentacular bulbs. Abnormal medusoids could break away from the mother colony because of a mechanical impact and move near the bottom of the experimental bowl. Abnormal medusoids were found on colonies of *Sarsia* sp. in the sea on 12 May 2021 (Figure S1, Table S1).

Analysis of COI

The analysis of the molecular phylogenetic tree and the haplotype net of the mitochondrial marker COI allowed us to divide the collected specimens into three species: *S. tubulosa*, *S. lovenii*, *S. princeps* (Figure 3, Table S2). Moreover, the specimens of *S. lovenii* form two haplogroups: haplogroup COI-1 and haplogroup COI-2 (Figure 3B; see also [13]). Haplogroup COI-1 includes specimens of morphotype I, III and IV (medusoid, attached medusa and abnormal medusoid). Haplogroup COI-2 includes specimens with morphotypes II and III (medusa, attached medusa).

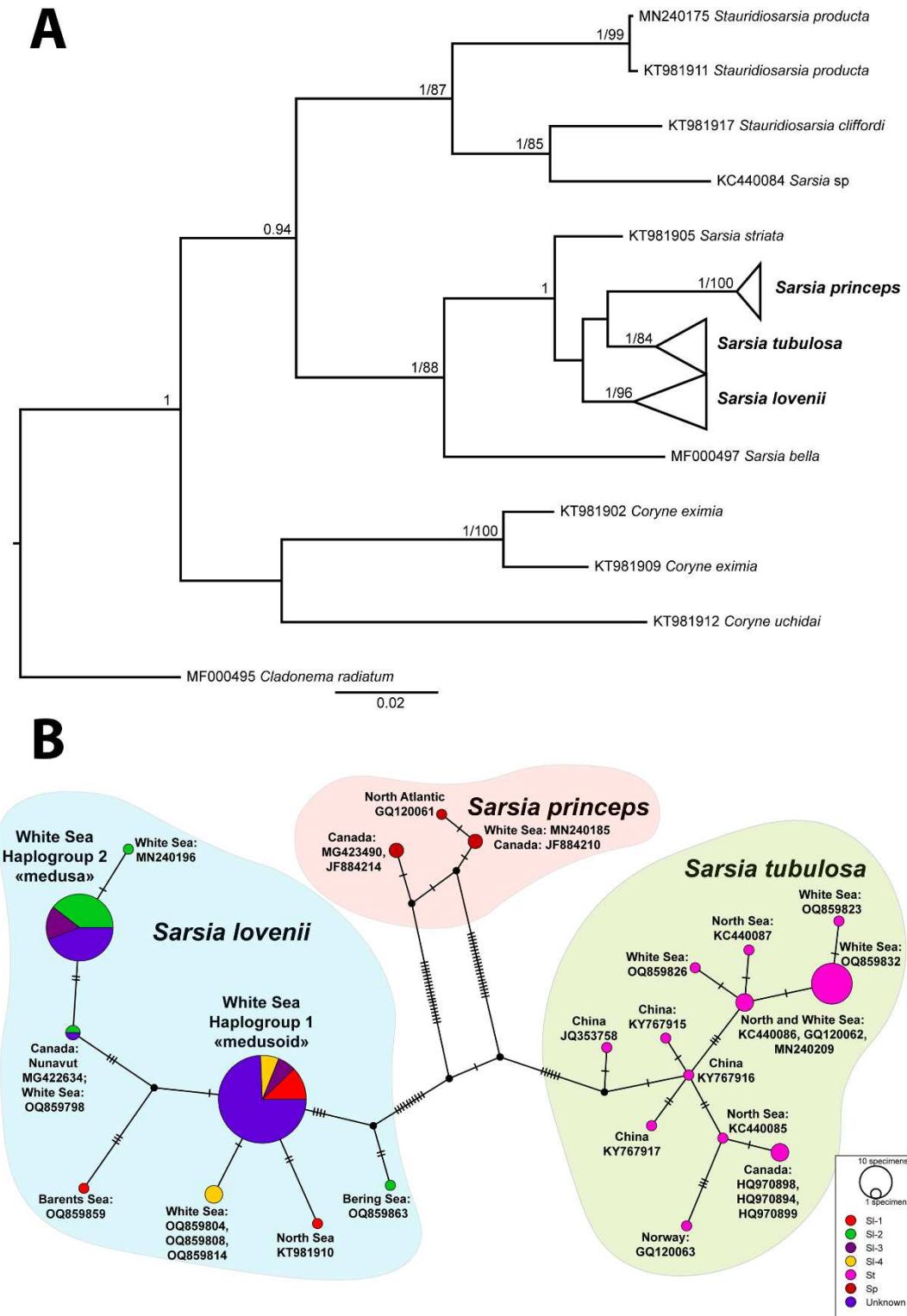


Figure 3. Phylogenetic analyses of COI-dataset: (A) Bayesian and Maximum Likelihood phylogenetic hypotheses. Numbers near branches show posterior probabilities (>0.95) and bootstrap values (>70). (B) Haplotype network. Abbreviations: morphotypes of gonophores *Sarsia lovenii* (Sl-1 – Sl-4) relate to morphotypes I-IV in the Figure 2. St – *Sarsia tubulosa*; Sp – *Sarsia princeps*; Unknown – specimens lack ripe gonophores.

Most specimens from the White Sea within each haplogroups have identical COI haplotypes. Some haplotypes of *S. lovenii* that have unique substitutions are mainly from other locations, such as the Barents Sea (OQ859859), North Sea (KT981910), Canada: Nunavut (MG422634). Specimen H248 (OQ859798) found in the deep-water part of the White Sea has also haplotype dissimilar from littoral

specimens but identical with medusa-specimen from Canada. We assign it to haplogroup COI-2 because the specimen from Canada is a medusa. There are also some unique haplotypes of *S. lovenii* collected in the shallow part of the White Sea, which are adjacent to one or another haplogroup. Medusa-specimen from the Bering Sea (H97) has unique haplotype (OQ859863) dissimilar with other medusa-haplotypes *S. lovenii*. Being closer to haplogroup COI-1, it nevertheless has the morphotype II. Specimens of *S. tubulosa* from the White Sea group with some haplotypes from the North Sea (Figure 3B). Specimens of *S. princeps* from the White Sea groups with some haplotypes from North Atlantic, including Canada's water and Iceland Sea (Figure 3B).

Analysis of ITS

For the analysis, a dataset with a length of 509 nucleotides was built. Within dataset, 31 positions were variable (Figure 4, Table S3). We distinguish groups of specimens belonging to certain haplotypes, as well as specimens with heterozygous states of certain loci, which may indicate the processes of hybridization between individuals with different haplotypes in populations.

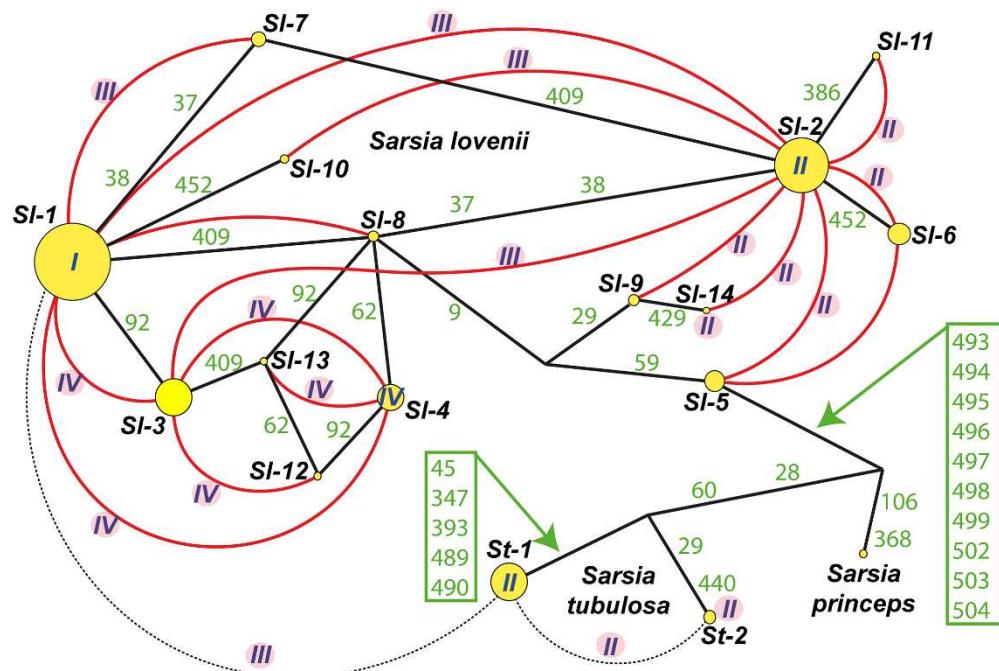


Figure 4. Haplotype network for ITS-dataset including *Sarsia lovenii*, *Sarsia tubulosa* and *Sarsia princeps*. List of specimens see in Table S4. Abbreviation: SI-1 – SI-14 – haplotypes *S. lovenii*, St-1 – St-2 – haplotypes *S. tubulosa*; I-IV – morphotypes of gonophores for haplotypes or hybrid specimens (I – medusoid, II – medusa, III – attached medusa and IV – abnormal medusoid); Green numbers – variable loci in ITS-dataset (see Table S3); Yellow circles – haplotypes ITS, size of circle relates to number of specimens; Black solid lines – connection between haplotypes; Red curves illustrate hybridization between haplotypes; The dotted line indicates hybridization between St-1 and St-2 haplotypes, as well as experimental interspecies hybrid between St-1 and SI-1.

Heterozygous specimens of *Sarsia tubulosa* have ITS-sequences with several single heterozygous loci. Such sequences could be the result of a combination of potential alleles, and the number of combinations increases rapidly with the increase in the number of heterozygotes. Therefore, we use only specimens of two main haplotypes without heterozygous loci (st-1 and st-2) in the network of haplotypes (Figure 4, Table S4). The differences between them were in seven loci. Heterozygotes from these loci found in 10 specimens probably indicate hybridization between lineages st-1 and st-2 (Figure 4, Table S4). Two haplotypes of *Sarsia tubulosa* and heterozygous specimens have gonophores of morphotype II (free-swimming medusa). We did not find any significant differences in the morphology of medusae of different haplotypes.

A large number of heterozygous loci were obtained for an experimental interspecies hybrid between *S. tubulosa* and *S. lovenii* (Figure 4: hybrid Sl-1/St-1). Due to the presence of several deletion zones, we could not determine the state of some loci when separating alleles by Champuru v. 1.1 [32]. Such hybrid colonies produced attached medusa (morphotype III) (DNA isolate H163: Figure S1, Table S1).

Several heterozygous specimens of *Sarsia lovenii* have ITS-sequences with group of double peaks in chromatograms. We managed to separate alleles of specimens with heterozygotes using Champuru v. 1.1 [32], as well as by cloning of two DNA isolates. As a result, we identified 14 haplotypes for *Sarsia lovenii* (Figure 4). Only three haplotypes (Sl-1, Sl-2 and Sl-4) include specimens having ITS-sequences without heterozygotes. The remaining specimens have ITS-sequences with heterozygotes and became part of different haplotypes, being divided into alleles. Hydroids with the Sl-1 haplotype produced normal medusoids (morphotype I). The Sl-2 haplotype was found in free-swimming medusae or in hydroids that produce free-swimming medusae. In addition, free-swimming medusae were registered in specimens with a mix of the allele Sl-2 and the alleles Sl-6, Sl-11, Sl-5, Sl-9, Sl-14.

A hybrid specimen was obtained in experimental crossing between the medusa with haplotype Sl-2 and the medusa with haplotype Sl-14 (DNA isolate H122). The resulted hybrid colony produced free-swimming medusae (morphotype II). Experimental hybridization of specimens with haplotypes Sl-1 and Sl-2 resulted in hybrid colonies that produced attached medusae (morphotype III) (Table 1). Colonies of hydroids with such a morphotype of gonophores were also found in the sea in May (DNA isolates H335, H337, H338). In addition, attached medusae were registered in specimens with a mix of the allele Sl-2 and allele Sl-10 or in mix of allele Sl-1 and allele Sl-7.

Colonies of hydroids with the haplotype Sl-4 produced medusoids of abnormal structure (morphotype IV). Several more heterozygous specimens for ITS had the same morphotype. Being separated into alleles, these sequences became part of the haplotypes: Sl-1, Sl-3, Sl-4, Sl-12, Sl-13 (Figure 4).

4. Discussion

4.1. Morphotypes of Gonophores in *Sarsia* spp. in the White Sea

In this work, we found four morphotypes of gonophores in *Sarsia* spp. (medusoid, free-swimming medusa, attached medusa and abnormal medusoid) compared to the three that were described earlier [13]. The structure of medusoids is quite variable. They differ in size, color, shape of the bell, the size of the manubrium and gonads. Morphotype IV, which we called "abnormal medusoids", differs from typical medusoids in the presence of short tentacles and a nematocyst zone at the tentacle bulbs (Figure 2D). On the other hand, abnormal medusoids do not have ocelli at tentacle bulbs and thus differ from attached medusae. This morphotype has not been previously described for *Sarsia* hydroids and, thus, it is not yet known whether it occurs outside the White Sea.

4.1. Species and COI-Haplogroups of *Sarsia* spp. in the White Sea

According to COI analysis, collected specimens can be attributed to three species: *S. lovenii*, *S. tubulosa*, *S. princeps* (Figure 3A). This result completely coincides with the previous analysis [13]. Analysis of haplotypes also recovered three groups that corresponds to three species. *Sarsia lovenii* specimens can be attributed to two main haplogroups. Previously, we divided these groups according to the morphotype of gonophores. Specimens with medusoid were assigned to haplogroup COI-1 and specimens with free-swimming medusa were assigned to haplogroup COI-2 [13]. However, here we demonstrated that each haplogroup includes specimens with several types of gonophores (Figure 3B). In each haplogroup there are specimens with an intermediate type of gonophore (morphotype III), which corresponds to hybrid forms. Given that mitochondrial genes are inherited on the maternal side, we believe that hybridization between haplogroups goes in both directions. In addition, haplogroup COI-1 also includes specimens with the morphotype of gonophore IV. A special position is occupied by a specimen from the Bering Sea, which has a medusa

as a gonophore, but is more closely related to haplogroup COI-1. Perhaps the medusa in the evolution of the species *S. lovenii* could be reduced to a medusoid and then recover again. However, to understand the phylogeography of the species *S. lovenii*, more specimens from different locations is required.

4.3. Hybridization or Intrageneric Polymorphism?

Nuclear ribosomal DNA (nrDNA) is the genomic region, in which the RNA components of ribosomes are encoded [35,36,37,38,39]. Eukaryotic nrDNA comprises a multigene family including transcribable rRNA genes (18S rRNA, 28S rRNA and 5.8S rRNA) separated by internal transcribed spacers (ITS1 and ITS2) and an intergenic spacer (IGS) that are located downstream of the 18S rRNA gene and upstream of 28S rRNA gene. These genes cluster in large tandems located on certain chromosomes to form nucleolus organising regions. Ribosomal genes and associated spacers are arranged in one or more large arrays consisting of hundreds or thousands of tandemly repeated copies. During evolution, the coding regions (18S and 28S rRNA) have remained more conserved than the non-coding regions (ITS and IGS). There is a considerable precedent for the use of ITS sequence divergence to infer relationships at or below the species level in a wide variety of taxonomic groups, most notably plants and fungi [40–43]. Sometimes ITS sequence were used in recovering phylogeny of cnidarian taxa such as corals [44] and hydrozoans [45–50]. In addition, ITS region is used to study intraspecific genetic heterogeneity [51]. In our study, we present a result of detailed analysis of the ITS marker in 183 specimens of *Sarsia* from the White Sea. We found three pure haplotypes of *S. lovenii*, two haplotypes of *S. tubulosa* and 1 haplotype of *S. princeps*. All these haplotypes did not contain heterozygotes. We also found intra-individual polymorphism in the structure of ITS for *S. lovenii* and *S. tubulosa*.

Two widely acknowledged problems with the usage of the ribosomal ITS region as a phylogenetic marker are intragenomic variation and alignment ambiguities [44]. ITS region can be hypervariable and prone to insertions and deletions, which can result in alignment ambiguities [44,51]. We have previously shown that ITS marker is suitable for the separation of haplotypes in *S. lovenii* [13]. However, when analyzing chromatograms, we encountered ambiguities of peaks in some specimens. While some of the substitutions, present in single specimens, may be PCR artifacts, the frequent occurrence of common patterns between specimens indicates that most of the sequence variations reflect real ITS heterogeneity. Since two main haplotypes of *S. lovenii* (Sl-1 and Sl-2) differ by deletion of two nucleotides (positions in the dataset 37–38), hybridization of these lineages results in hybrids with wide areas of double peaks in ITS chromatograms. The presence of parental ITS alleles in experimental hybrids was proved by cloning (sample H144) and by separation of alleles using Champuru software.

Tandemly arranged gene families tend to exhibit concerted evolution, a term used to describe the phenomenon when multiple copies of a gene family tend to be homogeneous, leading to greater sequence similarities among the paralogues within a genome than among orthologues among species [52,53]. Recombinant processes such as gene conversion and unequal crossover etc. are thought to be the homogenizing mechanisms [53–56]. Despite concerted evolution, intragenomic ITS variation has been found in many different types of invertebrates [57–62], indicating consideration has to be given for intra-individual rDNA variation. The simplest reason for the appearance of intra-individual rDNA variation is hybridization between different species or haplotypes of the same species [43]. Significant variation between copies within a species has been also attributed to introgression from hybridization, pseudogenes, separately evolving chromosomal lineages, slowed rates of lineage sorting of ancestral alleles [51,58,63–65]. Interspecific hybridization and intragenomic rDNA polymorphism are often difficult to distinguish [66].

We suppose that ITS polymorphism in *S. lovenii* and *S. tubulosa* is primarily associated with hybridization. Sequence data from ITS indicate that rDNA arrays are homogeneous in specimens related to haplotypes Sl-1, Sl-2, Sl-4 and St-1, St-2. Though we did not perform mass cloning of our DNA-samples, we assume that intragenomic polymorphism is absent or insignificant for these specimens. In addition, the polymorphism of many specimens might be explained by the presence of

hybrid forms between known haplotypes. Here, we experimentally proved that polymorphism is a result of crossing. Thus, questions remain for those specimens with ITS polymorphism for which we have not found potential parental haplotypes. Vegetative reproduction is likely to be a reason for maintenance of parental ITS sequences in the hybrids. It seems unlikely to us that intragenomic polymorphism occurs in some lineages of *S. lovenii*, but is absent in other lineages. However, the presence of a network of interconnected haplotypes in *S. lovenii* suggests the presence of genetic connectivity between them and the transfer of genetic material through recombinant processes. Thus, potential introgression due to hybridization, as a necessary component of reticulate evolution, may be an interesting direction for further research.

4.4. Hybridization Experiments

Here we experimentally confirmed the possibility of hybridization between different haplotypes of *S. lovenii*, as well as the possibility of interspecific hybridization between *S. lovenii* and *S. tubulosa* (Table 1, Figure S1). The possibility of hybridization between medusa and medusoid of *S. lovenii* has already been proven [13], but here we confirmed the result in several repetitions. We also performed a backcrossing between the first generation hybrid (F1) and the medusa *S. lovenii*. The crossing was successful. However, the poor survival of the resulting hybrids-F2 did not allow quantitative analysis of different alleles in the descendants. Nevertheless, our results confirm the possibility of such hybridization in the sea and support the possibility of introgression due to hybridization.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Photographs of specimens used for phylogenetic analyses. Full description of specimens presents in Table S1. Scale Bars 1 mm; Table S1: List of *Sarsia* specimens from the White Sea used for phylogenetic analyses: collection data and GenBank accession numbers. Abbreviations: Exp – Experiment. Species: Sl – *Sarsia lovenii*, St – *Sarsia tubulosa*. Sex: Fem – female, male. Gonophore type: 1 – medusoid, 2 – medusa, 3 – attached medusa, 4 – “abnormal medusoid”. Stage: p – polyp, m – medusa, pm – medusoid. Locality (see Figure 1): W1 – Aquarium at WSBS; W2 – Pier of WSBS; W3 – Eremeevskie rapids; W4 – saline lake at the Green Cape; W5 – location “Luda”; W6 – location “Krest”; W7 – Rugozerskaya inlet, depth 5-15 m; W8 – Polovye islands; W9 – Velikaya Salma Strait, depth 40-60 m. Table S2: Specimens, haplotypes and haplogroups COI of *S. lovenii*, *S. tubulosa* and *S. princeps* visualized at haplotypes net (Figure 3B). Sequences excluded from analysis of COI-haplotypes denoted in table as “exc”. Morphotypes for *S. lovenii*: medusoid, medusa, attached medusa, abnormal medusoid, unknown morphotype. Table S3: Alleles of significant loci of ITS dataset associated with interspecies and haplotypes differences (see Figure 4). Table S4: Specimens and ITS-haplotypes of *Sarsia lovenii* (Sl-1 – Sl-14), *Sarsia tubulosa* (St-1, St-2) and *Sarsia princeps* visualized at haplotype network (Figure 4). Abbreviations: allel_1 and allel_2 – haplotypes separated manually from sequences with one heterozygote. Phase1 and phase2 – haplotypes separated from sequences by means of Champuru v. 1.1 (Flot, 2007). St-1-add - specimens *S. tubulosa* of haplotype 1 with additive heterozygotes in some loci. St-1/2 – specimens with many single heterozygotes. Specimens of *S. tubulosa* with heterozygotes (St-1-add and St-1/2) were not included in haplotype analyses.

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