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New DNA sequence data on the enigmatic *Spirula spirula* (Linnaeus, 1758) (Decabrachia, suborder Spirulina)

E. Haring*, L. Kruckenhauser* & A. Lukeneder**

Abstract

The ram's horn squid *Spirula spirula* (Linnaeus, 1758) is a unique deep-water marine organism whose life cycle remains enigmatic. Interpretations of its ecology and habitat preferences are currently based solely on dredging, fishery data and isotope analysis. Molecular genetic analyses of dead specimens are one possibility to decipher phylogeographic questions of an otherwise unobservable deep-sea animal such as *S. spirula*. Here, we present new sequence data from a recently collected *S. spirula* specimen. In the three mitochondrial genes analysed, the distances between populations from the Atlantic and the Pacific are extremely low. Thus, molecular data do not suggest species differentiation within *S. spirula*.

Zusammenfassung

Das Posthörnchen *Spirula spirula* (Linnaeus, 1758) ist ein außergewöhnlicher Tiefseeorganismus, dessen Lebenszyklus nach wie vor Rätsel aufgibt. Das Wissen über Lebensweise und Habitat dieser Art basiert auf Aufzeichnungen aus der Fischerei, Aufsammlungen mittels Netz und Schleppnetz sowie auf Isotopenanalysen an Strandfunden. Molekulargenetische Analysen eröffnen eine Möglichkeit zur Klärung phylogeographischer Fragen bei Tiefseearten, die wie *S. spirula* der direkten Beobachtung nicht zugänglich sind. Funde von *S. spirula*, deren Gewebe analysierbare DNA enthält, sind jedoch sehr selten. In der vorliegenden Arbeit legen wir neue DNA-Sequenzdaten eines kürzlich gefundenen Individuums von *S. spirula* vor. In den drei mitochondrialen Genen, die analysiert wurden, sind die Distanzen zwischen den Proben aus dem Atlantik und dem Pazifik extrem niedrig. Eine Differenzierung der Gattung *Spirula* in zwei Arten, wie sie in der Vergangenheit diskutiert wurde, wird durch die vorliegenden Ergebnisse nicht gestützt. Zusammenfassend weisen die genetischen Daten auf fortgesetzten Genfluss zwischen Populationen von *S. spirula* und/oder auf relativ rezente Ausbreitung über große Distanzen hin. Für letztere Annahme fehlt jedoch noch eine schlüssige Erklärung für die Ausbreitungsmechanismen.

Key words: *Spirula spirula*, ram's horn squid, phylogeography, DNA analysis, deep-sea organism

Introduction

Knowledge on the ecology and life cycles of extant deep-water organisms is still poor. One example is the ram's horn squid *Spirula spirula* (Linnaeus, 1758) (Decabrachia, suborder Spirulina) a peculiar deep-sea organism whose lifecycle remains enigmatic. The species occurs in open subtropical to tropical oceans from about 30°N to 30°S (Clarke 1986, 1970, Okutani 1995, Haimovici et al. 2007, Lukeneder et al. 2008, Neige & Warnke 2010, Fig. 1). Interpretations of its ecology and habitat preferences are based

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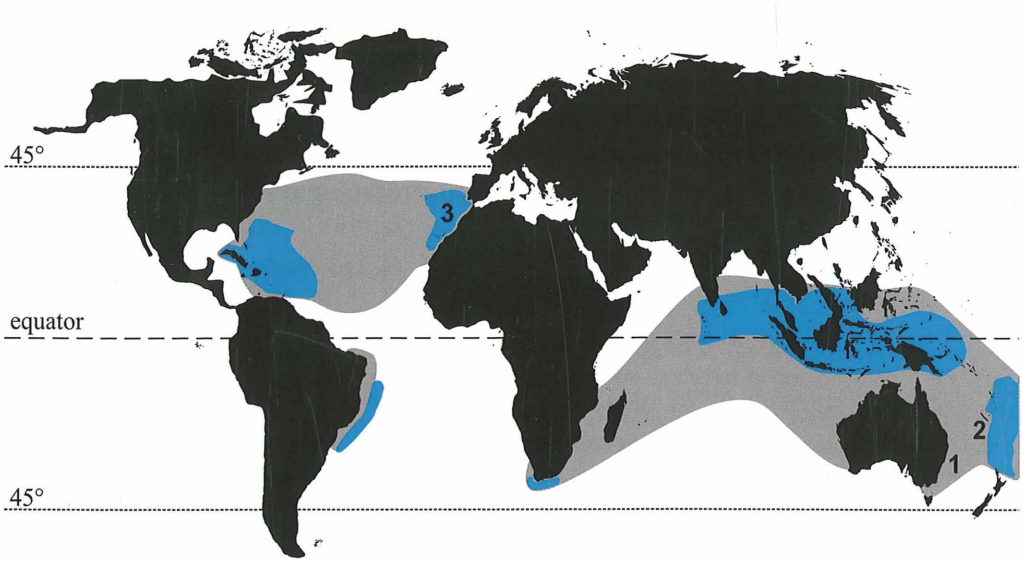

Fig. 1. Distribution of *S. spirula*. Blue areas indicate live catches. Dark grey regions mark shells found on beaches, by drifting, and fishery bycatch. Numbers 1–3 correspond to sites represented in the present comparative analysis. 1, Tasman Sea, Australia (this study), 2, Coral Sea, New Caledonia, France (BONNAUD et al. 1997), and 3, Canary Islands, Atlantic Ocean, Spain (WARNKE 2007). Distribution map compiled and modified after BRUUN (1943), GOUD (1985), SCHMIDT (1922), REID (2005), NORMAN (2007), LUKENEDER et al. (2008). For comparison see also OKUTANI (1995), HAIMOVICI et al. (2007), and NEIGE & WARNKE (2010).

solely on dredging and on fishery data (CHUN 1915, SCHMIDT 1922, KERR 1931, BRUUN 1943, CLARKE 1970, HAIMOVICI et al. 2007). *Spirula spirula* has a very long breeding season, with a peak during the winter months, and grows for somewhat more than a year until reaching sexual maturity (BRUUN 1943). CLARKE (1970) reported three different size-groups in *S. spirula* specimens caught by fishery with different types of nets around Fuerteventura Island (Canaries). Based on the numbers of spirulids captured over one year, CLARKE (1970) concluded that hatching probably took place in June to July and the squids grew to maturity after 12-15 months, when mating and egg laying took place. This suggested a protracted embryonic stage from October or December to the following June or July, and a life span of 18-20 months. The few captures in March indicated a reduced population at this time due to spawning, death, or movement into deeper waters. Sea-surface catches of drifting dead *S. spirula* shells were more numerous in March around Fuerteventura, suggesting increased mortality at that time.

Recently, LUKENEDER et al. (2008) showed that stable isotopes ($\delta^{18}O$, $\delta^{13}C$) in shells of dead specimens can help to decipher ontogenetic traits in these otherwise unobservable deep-sea animals. The exquisitely preserved shells provide a reliable geochemical archive that reflects the life span migration cycle of *S. spirula*. LUKENEDER et al. (2008) and PRICE et al. (2009) used the aragonitic internal shells as a biological proxy to interpret the ontogenetically related environmental changes of this model cephalopod. The result indicated an ontogenetically controlled vertical migration of the individuals.

After hatching in deep, cold seawater below 1000 m at temperatures around 4–6 °C, they start as a deep-water dweller during early ontogenetic stages but switch to warmer mid-water habitats (at 12–14 °C) during growth (LUKENEDER et al. 2008). Finally, fully grown adults tend to retreat once again to slightly deeper and cooler environments. The analyses indicated an identical mode of life for all specimens and excluded deviations in the vertical migration patterns between Atlantic and Pacific specimens. The resulting maximum range of temperature to which the squids are exposed during ontogeny is 9.1 °C. This reflects a bathymetric range of 800-1000 m for juveniles and a water depth of ~ 400-600 m (for temperature see LEVITUS94) in later ontogenetic stages. Besides these new insights into the vertical movements of *S. spirula,* nothing is known about lateral migration. The patchy distribution range of the species raises further questions about its phylogeography (Fig. 1). In this context, plate tectonics might provide a possible answer. Specifically, the formerly continuous distribution of *Spirula* may have been disrupted by the closing of the Paratethys in the Miocene, approx. 13–15 mya ago (HARZHAUSER & PILLER 2007, HARZHAUSER et al. 2007). In this case the Atlantic and Pacific populations should be genetically quite diverged. Note, however, that over the last few years new live catch data (SANTOS & HAIMOVICI 2002, PEREZ et al. 2004, HAIMOVICI et al. 2007, NEIGE & WARNKE 2010) showed that the species' distribution could be much wider than previously expected.

So far only a few individuals of *S. spirula* have been investigated genetically. Previous analyses focused on the systematic position of this species, although the results do not clearly resolve the phylogenetic relationships (BONNAUD et al. 1996, CARLINI & GRAVES 1999, LINDGREN et al. 2005). Some papers suggest that the Spirulidae may be the sister taxon of Sepiidae, but the respective branches in the trees are not well supported (CARLINI et al. 2000). WARNKE et al. (2011) estimated the divergence time between Spirulida and Sepiida at approx. 150 ± 30 million years ago (mya). Concerning intrageneric variation, WARNKE (2007) postulated that the genus *Spirula* comprises two species, represented by the geographically widely separated *Spirula* populations from New Caledonia (South Pacific) and Fuerteventura (Canary Islands). This hypothesis was based on the high difference obtained by comparing deduced amino acid sequences of the cytochrome oxidase III (COIII) protein (partial sequence of 218 amino acids). In that comparison the two sequences from Fuerteventura (WARNKE 2007) differed from the previously published sequence from New Caledonia (BONNAUD et al. 1996) by 3.9–4.6%. That study was based on three individuals only. However, considering that *Spirula* inhabits deep-sea environments and therefore targeted sampling is a rather challenging task. This makes it almost impossible to obtain the high numbers of specimens normally required for a comprehensive phylogeographic analysis; it also makes each single specimen an important source of information.

In their study on morphological variations in geographically separated *S. spirula* specimens NEIGE & WARNKE (2010) compared 110 shells of *S. spirula* from five geographical areas. They found a wide variation in characters of adult shells. Individuals from Madagascar, New Zealand and Brazil are larger than those from North-West Africa and Australia. The authors state that their results challenge the traditional monospecific status of *S. spirula*, but they also emphasize that these are only preliminary findings and that more molecular investigations are needed. In any case, the results presented to date are probably insufficient to claim species status for the two separated *S. spirula* populations.

In the present paper, we investigate a new *S. spirula* individual from the western Pacific collected at the eastern Australian coast in 2007. It was found with intact soft body (Fig. 2) and it was therefore possible to extract enough DNA to analyze three mitochondrial (mt) sequences: the protein coding genes cytochrome oxidase I and III (coI and coIII) and the 16S rRNA gene. The aim was to reassess the postulate of species differentiation between the populations from the Atlantic and the Pacific (Warnke 2007) and to interpret the genetic data in a phylogeographic context. As Neige & Warnke (2010) noted, the morphology in *Spirula* cannot operate as the sole proxy for proving the species types within this group of cephalopods. The molecular data presented here can help to understand these enigmatic deep-water squids.

Material and methods

DNA extraction & PCR amplification. The specimen of *S. spirula* (Spispi-1) analysed in the present study was collected in November 2007 in the Tasman Sea (near Avalon Beach, north of Sydney, New South Wales, Australia). The specimen is preserved in 70% ethanol and stored at the Natural History Museum of Vienna (2011/0233/0001). DNA was extracted using the DNAeasy blood and tissue kit (QIAGEN). Extraction was performed according to the manufacturer's protocol with a final elution volume of 100 µl elution buffer. A control extraction without tissue was done to test for contaminated reagents. Screening of GenBank for published sequences of *S. spirula* revealed a high number of nuclear genes (e.g., rhodopsin, octopine dehydrogenase, Pax-6) as well as several mt genes available for sequence comparisons. As we were especially interested

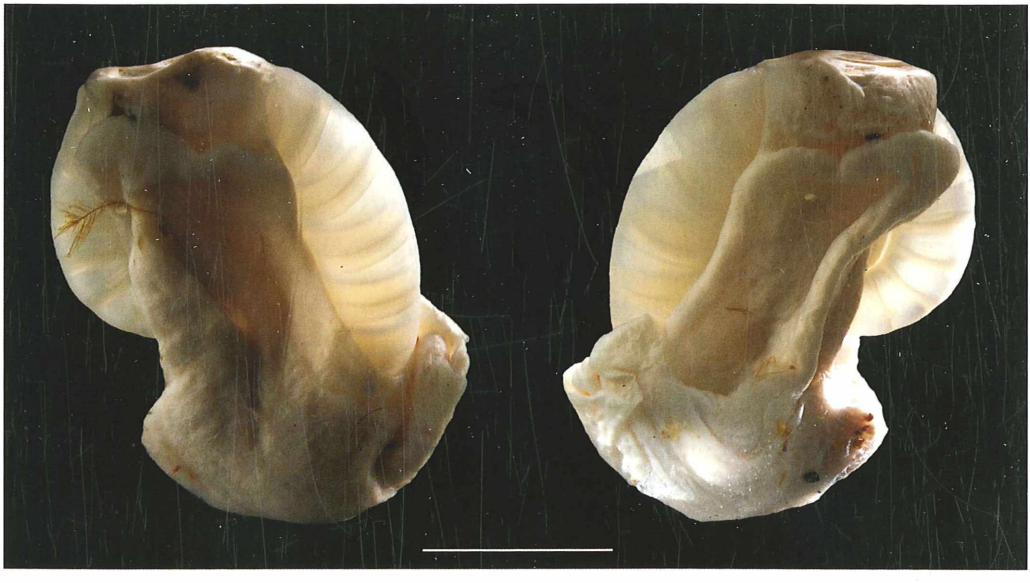

Fig. 2. *Spirula spirula* specimen from the Tasman Sea investigated in this study (Avalon Beach, north of Sydney, New South Wales, Australia; NHMW 2011/0233/0001). Note that the soft body is still attached to the entire internal shell. Front and back view of same specimen. Scale is 1 cm.

in population differentiation (i.e., intraspecific variation), we selected three mt genes that are available in GenBank from many cephalopods: (1) cytochrome c oxidase subunit I (coI), (2) cytochrome c oxidase subunit III (coIII), and (3) small ribosomal subunit rRNA (16S). PCR primers were either designed specifically for S. spirula based on published sequences or taken from the literature. Primer sequences for coI: LCO1490 5′-GTCAACAATCATAAAGATATTGG-3′ (FOLMER et al., 1994) and COIschneckrev 5′-TATACTTCTGGATGACCAAAAAATCA-3′ (DUDA et al., 2011). Primer Sequences for coIII: Spir-cox3-1-fwd 5′-CAATGATGACGAGATATTATYCG-3′ and Spir-cox3-1-rev 5′-GAACCATAAATGCTATCTGAG-3 (this study). Primer sequences for 16S: Spir-16S1+ 5′- ATCCAACATCGAGGTCGCA -3′ and Spir-16S2- 5′- TTTAACTG-TACTAAGGTAGC -3′ (this study).

The resulting fragment sizes were 707 bp (coI), 419 bp (coIII) and 380 bp (16S). PCR was performed on a Master gradient thermocycler (Eppendorf) in 25 µl with 0.2 units TopTaq (Qiagen), 1 µM of each primer, and 0.2 mM of each dNTP (Qiagen). Each PCR comprised 35 reaction cycles of denaturation at 94 °C for 20 sec., annealing for 30 sec., and elongation at 72 °C for 40 sec. Initial denaturation took place at 94 °C for 5 minutes, final extension at 72 °C for 10 minutes. The annealing temperatures were: 50 °C (coI), 54 °C (coIII) and 56 °C (16S). Control PCR reactions were performed with the control extractions and with distilled water instead of template. PCR products were purified with the Qiaquick PCR Purification Kit (QIAGEN) and sequenced directly (both strands) using the PCR primers. Sequencing was performed by AGOWA (Berlin, Germany).

Phylogenetic analysis. For each gene, additional *S. spirula* sequences were retrieved from GenBank for comparison (Fig. 3; Table 1) and included into the alignment. As outgroup we used, for all three sequences, *Sepia officinalis* LINNAEUS, 1758. Sequences were edited and aligned using the BioEdit software package version 5.0.9 (HALL 1999), only the *16S* sequences were aligned with t-coffee (NOTREDAME et al. 2000). In each alignment sequences were cut to those of the shortest length. Neighbour-joining (NJ) (SAITOU & NEI 1987) dendrograms were calculated with ClustalX 2.0.12 (LARKIN et al. 2007) using p-distances; for the *16S* sequences the gaps were excluded. The sequences determined during the present study are registered under the GenBank accession numbers listed in Table 1.

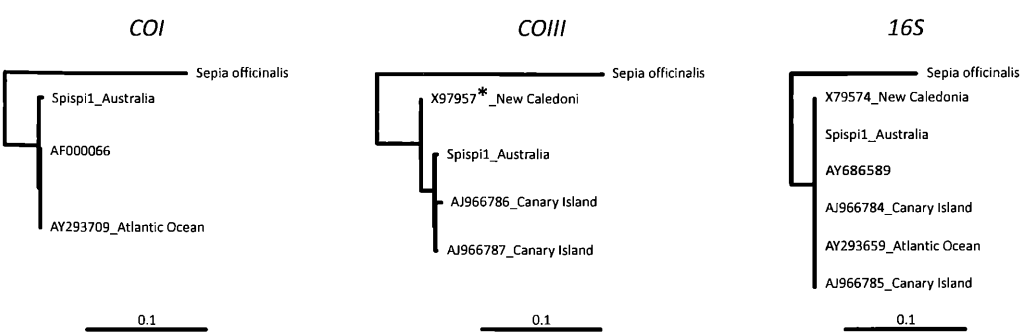

Fig. 3. NJ trees illustrating the genetic (p) distances in three mitochondrial genes between *S. spirula* specimens analysed so far. Geographic origins and GenBank Accession numbers of published sequences are indicated. For further details of specimens see Table 1.

Table 1. DNA Sequences of *S. spirula* and *S. officinalis* included in this study. Note: * = specimen Spispi-1 analysed in this study. References: 1, WARNKE (2007); 2, AKASAKI et al. (2006); 3, NISHIGUCHI et al. (2004); 4, CARLINI et al. (1999); 5, LINDGREN et al. (2005); 6, BONNAUD et al. (1994); 7, GIRIBET et al. (2006).

Species	Gene	Accession numbers	Reference	Geographic origin
*Spirula spirula**	COIII	JQ412174	this study	Avalon Beach, Australia
Spirula spirula	COIII	AJ966787	1	Canary Islands
Spirula spirula	COIII	AJ966786	1	Canary Islands
Spirula spirula	COIII	X97957	unpublished	New Caledonia
Sepia officinalis	COIII	AB240155	2	–
Spirula spirula	COI	AY293709	3	Atlantic Ocean
*Spirula spirula**	COI	JQ412176	this study	Avalon Beach, Australia
Spirula spirula	COI	AF000066	4	not specified
Sepia officinalis	COI	AF000062	4	–
*Spirula spirula**	16S	JQ412175	this study	Avalon Beach, Australia
Spirula spirula	16S	AY293659	3	Atlantic Ocean
Spirula spirula	16S	AY686589	5	not specified
Spirula spirula	16S	X79574	6	New Caledonia
Spirula spirula	16S	AJ966785	1	Canary Islands
Spirula spirula	16S	AJ966784	1	Canary Islands
Sepia officinalis	16S	DQ093491	7	–

Results

In the following, we describe the differences between coI, coIII, and 16S sequences isolated from the specimen Spispi-1 and published sequences of the three genes (see Table 1 for GenBank accession numbers). To illustrate the currently available data, we depict the relationships and distances among sequences in the form of NJ trees (Fig. 3).

There are two published coI sequences of *S. spirula* available in the GenBank database. One originated from the Atlantic Ocean (NISHIGUCHI et al. 2004; no detailed location given), the other one was published without geographic information (CARLINI & GRAVES 1999). Within the comparable section (657 bp) these two sequences are identical and differ from the Spispi-1 sequence at three positions (0.46 %).

Of the five 16S sequences of *S. spirula* published so far, three originated from the Atlantic Ocean [two from the Canary Islands (WARNKE 2007); one without further specification (NISHIGUCHI et al. 2004) and one from New Caledonia (BONNAUD et al. 1996)]. The comparable section spans 350 bp. The only differences within this region are two 1 bp-insertions in the sequence from New Caledonia and one autapomorphic transition in AJ966784 (Canary Islands). Thus, the maximum distance among the six sequences is 0.57 %.

Three coIII sequences of *S. spirula* are available in GenBank: two from the Canary Islands [AJ966786 and AJ966787; (WARNKE 2007)] and one from New Caledonia (X97957). The latter sequence is referred to as unpublished, although WARNKE (2007) provides the reference BONNAUD et al. (1994). The comparable section spans 369 bp. Comparing Spispi-1 with the sequences from the Canary Islands, only one base substitution is found between Spispi-1 and AJ966787 (we did not consider a purine at one ambiguous position in AJ966787 as a difference), while the maximum difference is three substitutions (between Spispi-1 and AJ966786; 0.81%). Two of these substitutions are transversions near the 3′end, one of them resulting in an amino acid replacement. The sequence from New Caledonia (X97957) is incomplete and deficient. It contains several undetermined positions (e.g., a section of 18 Ns in the middle of the sequence), two insertions and two deletions causing local frame shifts over a few codons. It also contains three transversions, which are usually much rarer than transitions in the mt genome. Both the transversions and the deletions are located close to the undetermined section and can thus be considered dubious. In the remaining parts, sequence X97957 is identical to Spispi-1 and AJ966787. As becomes clear from the alignment, sequence X97957 is deficient, and thus it appears meaningless to evaluate amino acid substitutions in comparison to the other sequences.

Discussion

In three mt genes (coI, coIII, 16S) the genetic distances among the sequences of *S. spirula* specimens from the Atlantic and the Pacific are very low (< 1 %). The observed p-distances are in the range expected for intraspecific variation; moreover, they are surprisingly low given the large geographic distances between the localities. Thus, the data presently available do no support the proposed split of *Spirula* into two species. Note also that DNA divergences alone are generally insufficient to prove species status, although they may be used to support specific hypotheses. Furthermore, the genetic separation between the Atlantic and Pacific populations was based on data from a single gene (coIII) that was translated in silico into a protein sequence. Importantly, the coIII sequence X97957 from New Caledonia, which was taken by WARNKE (2007) from GenBank for comparison, is incomplete and contains several dubious positions and insertions/deletions, causing frame shifts in the alignment. The proposed split into two species is therefore most probably based on artefacts. WARNKE (2007) did not outline how the amino acid sequence divergence of 3.9–4.6% was calculated, i.e., how the gaps and undetermined positions were treated, which makes it impossible to evaluate these distances. As a matter of fact, where local frame shifts occur due to insertions/deletions, stretches with completely different amino acids are obtained in the deduced protein sequence. As outlined above, however, we consider these sections in X97957 as being unreliable and the resulting differences in the deduced protein sequences as an artefact. Comparing the *coIII* sequence of our study with the two from the Canary Islands (WARNKE 2007) reveals only one amino acid substitution (methionine – leucine). Excluding the dubious sections of X97957, the DNA distances between all published sequences (X97957 and Spispi-1 from the Pacific, and the others from the Atlantic region) are surprisingly low (maximum 0.8%). Although data from the literature are scarce, some examples for intraspecific divergences in cephalopods are available. Groeneberg et al. (2009) investigated species of

the family Sepiolidae using a partial sequence of the mt coI gene as a molecular marker. They found very low intraspecific variation (< 1 %) in most species. Yet, compared to the situation in *S. spirula*, the geographic distribution range covered for those species is much smaller (North Sea). SINCLAIR et al. (2007) investigated the genetic diversity of isolated populations of *Nautilus pompilius* in the Great Barrier Reef, Australia, and the Coral Sea (localities 250 km apart) using a partial *coI* sequence (575 bp). They detected a clear differentiation of the two groups of populations (~ 2.3 %). The coI sequence was also used in the study of PÉREZ-LOSADA et al. (2007) on Atlantic and Mediterranean populations of *S. officinalis*. They reported significant divergence and low levels of gene flow within *S. officinalis* as well as interspecific sequence divergence up to 2.9 % between species of the genus *Sepia*. WARNKE et al. 2004 investigated the divergence within *Octopus vulgaris* CUVIER, 1797 and found divergence about 2% (16S) and 4% (coIII) respectively between the most diverged sequences (South Africa and Brazil). Samples from the Pacific (Taiwan and Japan) had slightly smaller distances to the two former localities, indicating that *O. vulgaris* occurs in the Atlantic as well as in the Pacific. In the light of these reports the genetic variation found within *Spirula* across huge geographic distances (Atlantic and Pacific populations) appears exceptionally low, contradicting the presumption of two *Spirula* species.

Do the genetic data obtained for *S. spirula* agree with the proposed phylogeographic history of the species? Assuming the closure of the Paratethys in the Miocene [approx. 13-15 mya; (HARZHAUSER & PILLER 2007, HARZHAUSER et al. 2007)] as an isolating factor separating the Atlantic and Pacific populations, the expectation would be that population divergence is reflected by diversification at the DNA level. HOU et al. (2011) could show diversification shifts in crustaceans which correspond to the regression of the Paratethys and as the authors noted an increasing continentalization of Eurasia co-occurring with an isolation of different oceans (e.g., Paratethys, Mediterranean Sea) in the Miocene. See also the work on adaptive radiation by GAVRILETS & LOSOS (2009). However, the populations of *Spirula* are not genetically distinct: the representatives are identical or almost identical in the three marker sequences. Even assuming extremely low evolutionary rates, the distance values in mt genes for two groups isolated for such a long time would be expected to be much higher. This clearly rules out this explanation. One possibility is that, initially, a widely distributed ancestral population of *S. spirula* was split up with the closure of the Parathethys, but later the population at one side of the new barrier became extinct. Nonetheless, although the number of individuals from whom genetic data are available is still very low, the data suggest that present-day *S. spirula* populations in the Pacific and the Atlantic have a rather recent common origin.

Which scenarios can explain the low genetic differentiation over such long distances? (1) One possibility is long-distance dispersal, which again should have occurred quite recently. From which population did the dispersal start and which factors mediated it? The short life span [e.g., approx 18-20 month; (CLARKE 1970, LU et al. 1992, LUKENEDER et al. 2008)] of the individuals makes it even more difficult to draw any plausible scenario involving long-distance dispersal.

Transportation by human activity (e.g., ships) can be excluded because the animals live in deep sea and depend on low temperatures. Eggs might drift with strong oceanic deep-sea currents (e.g., great ocean conveyor belt), although none of the known deep ocean

currents fits the observed distribution of *S. spirula*. However, deep-sea currents in the late Pleistocene / early Holocene might have been different. This leads to a second explanation that assumes the split of a former huge population in the recent past (e.g., postglacially), resulting in disjunct distribution areas. Recent findings on the distribution of *S. spirula* favour a third explanation: a (so far) undetected huge panmictic population with continuous gene flow. Live-catch data accompanied with reported drift-shells washed on beaches (BRUUN 1943, CLARKE 1986, GOUD 1985, LUKENEDER et al. 2008, NORMAN 2007, OKUTANI 1995, SCHMIDT 1922, REID 2005) resulted in various different distribution maps. New data from Brazil gained by trawling and bycatch (PEREZ et al. 2004, HAIMOVICI et al. 2007) and by investigating stomach contents of fishes and other squids [e.g. diverse teleosts and *Illex argentinus* (CASTELLANOS, 1960); see SANTOS & HAIMOVICI (2002)] showed that the distribution of this deep-water squid could be much wider than expected on the basis of previous data. Accordingly, the populations might have some connections in mid- and deep-water areas around the world's oceans. Nonetheless, even in this case the low genetic distances observed over such a broad geographic range are surprising.

In summary, genetic data, even though scarce, suggest continuous genetic exchange among *S. spirula* populations and/or quite recent long-distance dispersal, although the latter assumption still requires a conclusive explanation for the mediating mechanisms. More data from deep-water live catches and additional molecular results are needed to support our interpretations.

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