Chapter 7
On the Species Status of *Spirula spirula* (Linné, 1758) (Cephalopoda): A New Approach Based on Divergence of Amino Acid Sequences Between the Canaries and New Caledonia

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1 Introduction

Spirula is one of the most unusual Recent cephalopods, with a unique chambered shell related to an osmotically regulated buoyancy control, a unique photophore at the tip of the mantle, an oegopsid eye, and a greatly reduced radula (Nixon and Young, 2003). The external structure of the early *Spirula* shell starting from a spherical initial chamber resembles very much that of Ammonoidea, indeed more than that of Belemnoidea (Bandel and Boletzky, 1979; Bandel, 1982). Thus, *Spirula* is likely to provide interesting insights concerning both biology and paleontology.

Well preserved material of *Spirula* is scarce, however, despite the countless shells found on oceanic beaches (Dauphin, 1979a, b). As a consequence, the question whether there is more than one species of *Spirula* has not yet been answered definitively. The chief aim of the present study is to discuss the reliability of molecular data for a distinction of inter- versus intraspecific divergence of *Spirula*.

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2 Taxonomy

In early papers on *Spirula*, up to five species were described (Owen, 1879; Huxley and Pelseneer, 1885; Lönnberg, 1896). Only one species was accepted by Chun (1915), Naef (1923), and Bruun (1943). Nesis (1987) and Norman (2000) also described one living species of *Spirula*. A major problem in the description of more than one species of *Spirula* is that different "species" are represented by only a few, often incomplete specimens. Moreover, most type repositories are unknown (Young and Sweeney, 2002). At present, the taxonomic status of *Spirula* is still called into question. Indeed, Young and Sweeney (2002) listed most species of *Spirula* as either undetermined or *nomina nuda*. Young and Sweeney (2002) considered only *Spirula spirula* as a valid species (Table 7.1). Nonetheless, the type repository of this holotype remains unknown and the type locality is extremely vague.

No doubt, *Spirula* is widely distributed. Live specimens occur in the waters of Indonesia, Melanesia, Australia, southeastern Africa, between the Canary Islands and northwestern Africa (Fig. 7.1), in the Caribbean Sea, and in the Gulf of Mexico (Bruun, 1943; Clarke, 1966; Dauphin, 1979a, b; Nesis, 1987).

Apparently the geographic range of *Spirula* is disjunct like that of many other epi- and mesopelagic, circumtropical species that are widely distributed in the Atlantic and Indo-West Pacific. Probably there is no gene flow between the Atlantic and Indo-Pacific populations living around South Africa (Nesis, 1998). Thus, one can infer the occurrence of geographic subspecies, one in the Atlantic and the other in the Indo-West Pacific (Nesis, 1998). Subspecies are rarely used in cephalopod systematics (Voss, 1977). However, Bruun (1943) already considered the

Table 7.1 *Status of* Spirula *species after Young and Sweeney (2002).*

Species name	Determination	Type locality	Comments
Spirula peronii atlantica	[*forma*] Girard, 1890: 250.	Algarve (Portugal); Azores; etc.	Undetermined
Spirula australis	Lamarck, 1816: pl 465, Figs. 5a, b.	Not designated	Undetermined
Spirula blakei	Lönnberg, 1896: 100.	West Indies	Undetermined
Spirula fragilis	Lamarck, 1801: 102.		Nomen nudum
Spirula peronii indopacifica	[*forma*] Girard, 1890: 250.	New Caledonia; Indian Ocean; etc.	Undetermined
Spirula peronii	Lamarck, 1822 in 1815–1822: 601.	"l'Océan austral et celui des Moluques"	Undetermined
Spirula prototypus	Lesueur and Petit, 1807: pl 30, Fig. 4.	Not designated	Undetermined
Spirula reticulata	Owen, 1848: 14, pl. 4, Figs. 3, 9.	Off Timor [*fide* Lönnberg (1896: 99)]	Undetermined
Loligo spiralis	d'Orbigny, 1826: 153	–	Nomen nudum
Nautilus spirula (Spirula spirula)	Linné, 1758: 710 [*fide* Bruun (1943: 3)]	"America"	Valid species

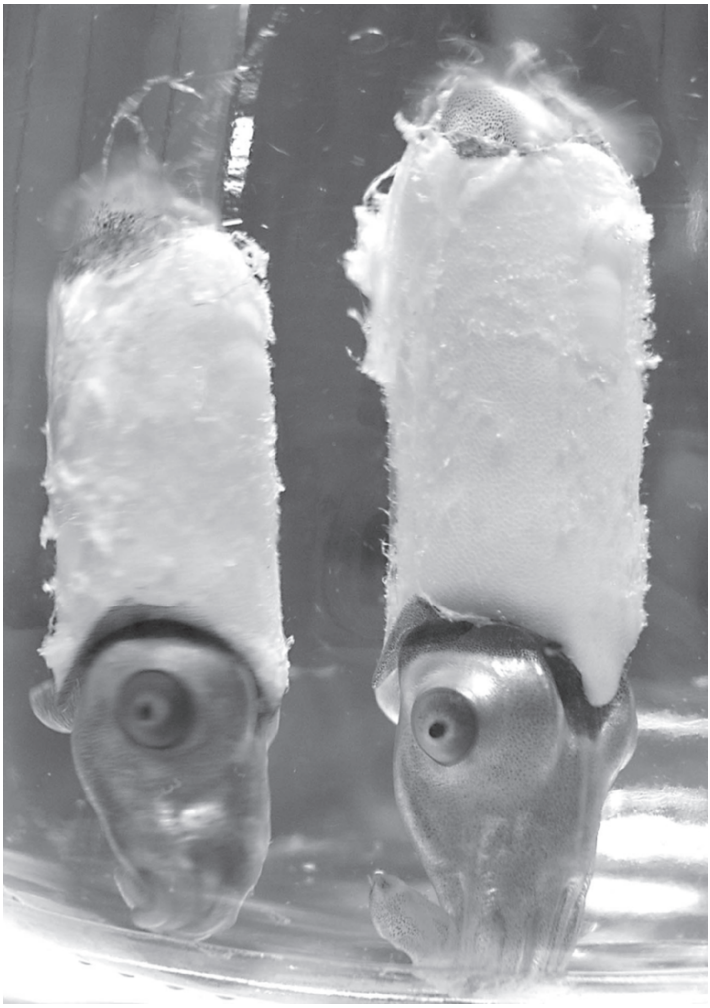

Fig. 7.1 *Living* Spirula spirula *(left, female [mantle length = 4 cm], right, male [mantle length = 5 cm) caught between the Canary Islands and the coast of Morocco. The animals were caught with an Isaacs Kidd mid-water trawl and placed in an aquarium to take photos.*

possibility of subspecies or races of *Spirula* based on morphological characters. He compared preserved soft parts from the Atlantic and the Indo-Pacific and analyzed them with regard to pigmentation, size, sexual arm differentiation, similarity of external characters, and some further morphometric measurements, but he did not find any definite morphological difference between them.

Nesis (1998) again emphasized the potential importance of biochemical or molecular data to distinguish intra- and interspecific differences, especially regarding species with a distribution pattern like that of *Spirula*.

3 DNA Sequence Data

In most cases, molecular data provide informative phylogenetic indications for determining evolutionary relationships between sibling species or at least morphologically nearly identical species. A famous example is the study of teleost fish species by Sturmbauer and Meyer (1992). Numerous, nearly morphological identical cichlid species recognized in Lake Tanganyika are genetically divergent. The genetic divergence within these species displays twice as much as the genetic divergence within the morphologically different cichlid species from Lake Malawi. Many further examples can be found in the literature of the last 20 years (e.g., Palumbi and Benzie, 1991; Knowlton et al., 1993).

Using allozyme markers, several studies have been carried out to detect the genetic divergence of especially closely related cephalopod species, cryptic speciation, and population structure (Augustyn and Grant, 1988; Levy et al., 1988; Carvalho and Pitcher, 1989; Brierley et al., 1993; Katugin, 1993; Yeatman and Benzie, 1993; Allcock et al., 1997; Pérez-Lozada et al., 1999; Maltagliati et al., 2002). Using randomly amplified polymorphic DNA markers (RAPD) or restriction fragment length polymorphisms (RFLPs), the genetic distinction between cephalopod species or between subunits inside the species themselves can also be established (Warnke et al., 2000; Herke and Folz, 2002; Chapela et al., 2003; Sands et al., 2003). Shaw (2002) reviewed all these papers as well as the studies based on microsatellite markers, which are used to analyze the population structures of cephalopods.

The DNA sequence data used for direct analyses were mostly obtained from mitochondrial genes. The advantage of mitochondrial DNA (mtDNA) over nuclear DNA is that this small extra-nuclear part of the genome can be found in multiple copies. Consequently, a small amount of tissue (e.g., 10–15 mg from the mantle) is sufficient to isolate the required initial amount of DNA for PCR-based methods. In the present case, this constitutes a major advantage, since only few freshly caught animals were available and they would not have to be destroyed so that they could be used for further morphological studies. MtDNA is known to evolve relatively fast; it is still used widely today to assess taxonomic relationships and differences between populations within species. MtDNA sequences provide the most direct means for measuring genetic diversity to determine the sequences of bases in the DNA (Beebee and Rowe, 2004; Frankham et al., 2004). MtDNA also encodes for rRNA. The 16s rRNA gene has one further advantage in that universal primers are available (Kocher, 1992).

Based on mtDNA sequences, several studies have been devoted to the inter- and intraspecific variations within Recent Cephalopoda. MtDNA sequence data were used by Wray et al. (1995) among others. These authors traced the geographically related diversifications of some genetically distinct lineages of *Nautilus* using a portion of the mt16S rRNA gene. Molecular data were also used to check genetic relationships within the genus *Octopus* (Barriga Sosa et al., 1995; Hudelot, 2002). Other studies examined in particular the distribution of *O. vulgaris*. Using mtCOIII

(cytochrome oxidase subunit III) and mt16S rDNA sequences (Warnke, 1999; Söller et al., 2000; Warnke et al., 2002, 2004), the distribution of *O. vulgaris* was confirmed for the Mediterranean as well as for the eastern and western Atlantic. *Octopus vulgaris* could not be found in the eastern Pacific.

Studies concerning interspecific variations in the genus *Pareledone* were performed by Allcock and Piertney (2002) again, using mt16S rDNA sequences. During study of higher-level relationships of coleoids, intra- or interspecific sequence variations of Decabrachia and Vampyropoda were also analyzed as well (Bonnaud et al., 1994; Boucher-Rodoni and Bonnaud, 1996; Bonnaud et al., 1996, 1997; Carlini and Graves, 1999; Carlini et al., 2001). For example, the sequence divergences between two different species of the genus *Loligo* were examined focusing on partial sequences of the mitochondrial 16S rDNA and COIII (Bonnaud et al., 1996). Analysis of the phylogeny and biogeography of loliginid squids was made by Anderson (2000) based on mtDNA (COI, 16S) sequences. Nishiguchi et al. (2004) analyzed the phylogentic relationships between Sepiolidae species using mtDNA (COI, 12S, 16S) and a partial sequence of a nuclear gene (28s rRNA).

The phylogenetic position of *Spirula* within the Decabrachia is still called into question, but a position next to *Loligo* or next to *Sepia* is discussed (Bonnaud et al., 1996; Warnke et al., 2003; Strugnell et al., 2005). Genetic studies within the genus *Sepia* are less frequent. For example, Zheng et al. (2001) described the genetic variation within the common Chinese cuttlefish *Sepiella maindroni* using the cytochrome oxidase subunit I (COI).

To date, no molecular investigation of intra- or interspecific variations of *Spirula* has been made. Therefore, a preliminary molecular analysis of *Spirula* is presented in order to obtain some clues about whether there is more than one *Spirula* species. MtCOIII and mt16S rDNA were used in this analysis because corresponding sequences of *Spirula* from a different part of their distribution area already exist in the EMBL data bank, and because these sequence data proved useful earlier on in determining evolutionary relationships between nearly indistinguishable sibling species.

4 Material and Methods

Live animals of *Spirula* were caught near Fuerteventura (Canary Islands, Spain, Fig. 7.2). They were preserved in 95–100% ethanol. A small tissue sample was taken from the arm tip of each animal. DNA was isolated from these samples following the Chelex method (Walsh et al., 1991) modified by Söller et al. (2000). Chelex supernatant was purified with the DNeasy Kit (Quiagen, Hilden).

A fragment of mitochondrial ribosomal 16S RNA gene (16S) and the cytochrome oxidase III gene (COIII) were used as target sequences. The DNA of mt16S rDNA was amplified by PCR using universal primers 16Sar and 16Sbr (Simon et al., 1991). For the COIII fragment, primers from Barriga Sosa et al. (1995) and Warnke

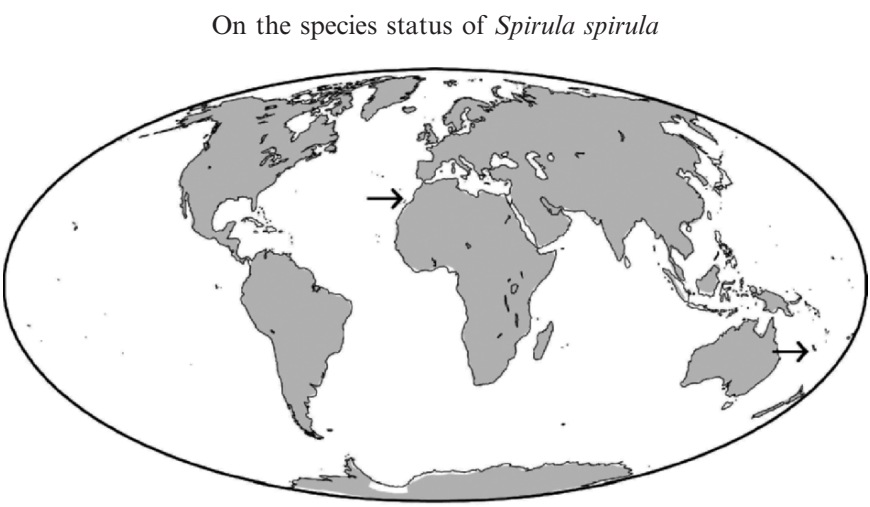

Fig. 7.2 *Map of localities of* Spirula *sample (arrows show sample location, map modified after Scotese, 2001).*

(1999) were used. These primers were designed for obtaining the DNA of COIII from various *Octopus* species. A polymerase chain reaction (PCR) was performed in 50 µl reaction volumes containing 10 mM Tris-HCL, pH 8.8; 25 mM KCL; 2 mM $MgSO_4$; 0.2 mM each of dATP, dCTP, dGTP, dTTP; 0.2 µM of both forward and reverse primers; 0.5 U *Taq* polymerase (Pharmacia Biotech, Freiburg i. Br.), 1–10 µl DNA solution (purified Chelex supernatant).

The fragments were sequenced and arranged together with three further *Spirula* sequences from the EMBL database [EMBL Acc-Nr.: X79574, Bonnaud et al. (1994), fragment of mt16S DNA; EMBL Acc-Nr.: X97957, Bonnaud et al. (1996), fragment of mtCOIII; EMBL Acc-Nr.: AY293659, Nishiguchi et al. (2004), fragment of mt16S DNA)] in a multiple sequences alignment. The *Spirula* specimens used by Bonnaud et al. (1994, 1996) were recovered from New Caledonia. For the animal mentioned by Nishiguchi et al. (2004), the Atlantic Ocean was given as the collecting site. The sequences of this study have been deposited in the EMBL database (accession numbers follow: AJ966784 *Spirula spirula* isolate 1, fragment of mt16S DNA; AJ966785, *Spirula spirula* isolate 2, fragment of mt16S DNA; AJ966786, *Spirula spirula* isolate 2, fragment of mtCOIII; AJ966787, *Spirula spirula* isolate 1, fragment of mtCOIII). The sequences of the animal 1, 3, and 4 were identical. Because of this, just the sequence of animals 1 and 2 were deposited in the database.

Sequence divergences were examined using the PAUP* program (Swofford, Smithonian Institution, Washington, DC). Additionally, the nucleotide sequences of COIII were translated into the amino acid sequence using the invertebrate mitochondrial code. The putative phylogenetic relationships were calculated applying

both distance- and character-based analyses of the data. The trees were rooted with *Octopus vulgaris* (EMBL Acc-Nr.: AJ012121, Söller et al., 2000) as an outgroup representative.

5 Results

From each sample, fragments of up to 500 base pairs of mt16S rDNA and additionally 500 base pairs of COIII were amplified by PCR. The sequence divergence of 16S was zero or nearly so (0–0.2%) for all animals. The nucleotide divergence detected for COIII was also relatively low between populations of the Canary Islands (0–0.4%). The divergences between the nucleotide sequences of *Spirula* from Fuerteventura and New Caledonia were high (0.7–1.3%). However, all these substitutions were transversions. Transversions are mutations in which a purine nucleotide (Adenin, Guanin) is replaced by a pyrimidine nucleotide (Thymin, Cytosin). Compared to transitions, transversions do not occur often. Transitions are mutations in which a purine is substituted by the other purine nucleotide or a pyrimidine nucleotide by another pyrimidine (Beebee and Rowe, 2004). Moreover, when comparing the amino acid sequences of this gene (as obtained by the invertebrate mitochondrial code), the divergence between *Spirula* populations from the Atlantic and the Pacific increased considerably to reach a value of 3.9–4.6% while the divergence within the population of Fuerteventura remained low (0–0.66%). Calculation of an MP tree was not possible. PAUP found only two informative characters. These are not sufficient for a reliable parsimonious analysis (Hillis and Huelsenbeck, 1992).

6 Discussion

The relatively high divergence between the *Spirula* amino acid sequences of animals from New Caledonia and Fuerteventura suggests that the genus *Spirula* consists of more than one species. Using the gene COIII, the mentioned divergence is normally found among different species of cephalopods (Barriga Sosa, 1995; Bonnaud et al., 1996; Warnke, 1999; Söller et al., 2000). This observation notwithstanding, it is unusual to find such an enormous difference between the amino acid sequences of COIII. The limited data of this study are not unambiguous, however, in particular because only one sequence is available for *Spirula* from New Caledonia. Furthermore, it seems desirable to get more *Spirula* from distribution areas to conduct more detailed morphological investigations. In addition to the scarcity of morphological and molecular data, a discussion of the distribution of *Spirula* is difficult because data about spawning and the mode of life of *Spirula* hatchlings are not available (Bruun, 1943; Clarke, 1966). In particular, we know nothing about how far the paralarvae may drift in the open sea. But in general terms, it

seems unwise to assume a recent gene flow between *Spirula* populations of the Canary Islands and of New Caledonia because *Spirula* has just a short life span of about 20 months (Clarke, 1970). Knowlton (2000) emphasized that the tools of molecular genetics have a great potential for clarifying the species boundaries in marine organisms. As compared with other molluscs or different invertebrates, cephalopod species boundaries have been examined rather little, so much remains to be done.

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