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ORIGINAL ARTICLE

Sipunculus nudus Linnaeus, 1766 (Sipuncula): cosmopolitan or a group of pseudo-cryptic species? An integrated molecular and morphological approach

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Abstract

Sipunculan taxonomy relies on a limited set of external morphological and internal anatomical characters. In addition, this marine group is characterized by an unusual large number of putatively cosmopolitan species. However, this 'cosmopolitan' status could be an artifact of their conserved morphology and the small number of unambiguous taxonomic characters available for delimiting species. Species delimitation can therefore be aided by molecular techniques. We investigated the case of the widespread and common species *Sipunculus nudus* Linnaeus, 1766 to determine its systematic validity. We analysed the morphology of multiple specimens of *S. nudus* collected from 11 localities around the world and undertook phylogenetic analyses using molecular sequence data from four genes (28S rRNA, 16S rRNA, histone H3 and cytochrome *c* oxidase subunit I). High levels of genetic differentiation are present between distantly related populations of the putative species *S. nudus*. Five distinct lineages were identified by phylogenetic analyses, three of which – the best-represented populations – can be distinguished morphologically. Our phylogenetic and morphological analyses thus do not favor the cosmopolitan status of *S. nudus*, suggesting instead that it constitutes a complex of morphologically similar but distinguishable species.

Introduction

Cosmopolitanism is a concept attributed to species that are distributed throughout the world (Spellerberg & Sawyer 1999). Among marine species with simple morphologies and few diagnostic characters, this concept can be nebulous, hindering failures in differentiating closely related species (Knowlton 1993; Thorpe & Solé-Cava 1994; Klautau *et al.* 1999). Consequently, this problem of morphological taxonomy may have led to what Klautau *et al.* (1999) defined as a worldwide 'lumping' of many morphologically similar, but evolutionarily distinct, species into single, artificially cosmopolitan morphospecies. This is often the case in sipunculans (Fig. 1), a group of exclusively marine invertebrates, where a large proportion of species known from multiple localities are considered cosmopolitan and where, often, the term is loosely applied to any species, with a range spanning at least the width of an oceanic basin (Schulze *et al.* 2012).

Over the last three decades molecular studies have helped to reveal cryptic species in many groups of marine soft-bodied invertebrates with few diagnostic morphological characters (*e.g.* Solé-Cava & Thorpe 1986; Knowlton 1993, 2000; Klautau *et al.* 1999; Thornhill *et al.* 2008; Barroso *et al.* 2010; Andrade *et al.* 2011). Conversely, studies that confirm cosmopolitanism among marine benthic invertebrates are rare (see Nóbrega *et al.* 2004; Ahrens *et al.* 2013), and the few focusing on sipunculans seem to refute cosmopolitanism.

Cosmopolitanism in Sipuncula was investigated for the first time by Staton & Rice (1999) in *Apionsoma misakianum* (Ikeda, 1904), a species widespread throughout the

Fig. 1. Three morphologically similar species of large sipunculans from the intertidal zone of Fort Pierce, Florida (USA). On the left: *Siphonosoma cumanense* (Keferstein, 1827), in the center: *Sipunculus nudus* Linnaeus, 1766, and on the right: *Xenosiphon branchiatus* Fisher, 1947.

Western Atlantic, Indo–Pacific, and Eastern Pacific oceans. Using allozyme data, they showed evidence for cryptic speciation, questioning the cosmopolitan status of this species. Du *et al.* (2008, 2009) undertook two isolated population genetic studies, one based on 16S ribosomal RNA (henceforth 16S), and a second study using cytochrome *c* oxidase subunit I (henceforth COI). For both studies they sequenced individuals from the same three populations of what they identified as *Sipunculus nudus* Linnaeus, 1766 from Southern China. Although considered cosmopolitan, these studies show significant variability and genetic structure among the three geographically close populations. Kawauchi & Giribet (2010) evaluated the validity of the circumtropical *Phascolosoma perlucens* Baird, 1868. Using two regions of mtDNA (16S rRNA and COI) and morphological data, that study suggested that *P. perlucens* is a complex of species, and its purported cosmopolitanism was a consequence of taxonomic tradition causing lumping. More recently, Schulze *et al.* (2012), using a molecular approach combined with morphological and developmental data, demonstrated that three other widespread species, *Phascolosoma agassizii* Keferstein, 1866, *Thysanocardia nigra* (Ikeda, 1904), and *Themiste pyroides* (Chamberlin, 1920), also represent complexes of cryptic or pseudo-cryptic species.

A series of recent multilocus approaches to sipunculan phylogeny corroborated the existence of cryptic (two or more species to be classified as a single nominal species because they are morphologically indistinguishable; Mayr 1948), or pseudo-cryptic (a species that can be readily identified by key morphological characters after a detailed comparison of morphological and non-morphological features; Sáez & Lozano 2005) species. The analyses performed by Maxmen *et al.* (2003), Schulze *et al.* (2005, 2007), and Kawauchi *et al.* (2012), including multiple representatives of putatively cosmopolitan sipunculan species from different ocean basins, showed that several species are probably not monophyletic. Among these we find the large and conspicuous *S. nudus*, which exhibits an exclusive suite of characters that make this species unique among other sipunculans (Cutler 1994). According to Cutler (1994), the most striking difference observed in the species is its developmental biology, since *S. nudus* is the only sipunculan to have micromeres smaller than the macromeres, and to gastrulate exclusively via invagination. Rice (1988) described that *S. nudus* from Puerto Rico has particular features of the early larval development, such as a unique ciliation pattern and the fate of the egg envelope. From an anatomical point of view, the nerve cord, with a swollen bulb on the posterior end, is unique to this species, and other exclusive differences are the coelomic urn cells, regeneration capabilities, osmoregulation, and chromosome number (Cutler 1994).

With a considerable size (5–15 cm long), *S. nudus* was named by Linnaeus, described from 'European waters' (Linnaeus 1766). Many subsequent species were assigned to this genus, but during the 20th century most were either transferred to other genera as new higher taxa were established, or became junior synonyms of *S. nudus* (Stephen & Edmonds 1972; Cutler & Cutler 1985; Cutler 1994). This process resulted in the lumping of about nine previously described species (Saiz-Salinas 2013), and the subsequent cosmopolitanism, as the lumping included samples from tropical to temperate

waters, from the intertidal to 900 m deep, and in all ocean basins (Cutler 1994).

In the first monographic treatment of Sipuncula, Stephen & Edmonds (1972) examined multiple individuals from the Atlantic coast of France and assigned diagnostic morphological characters to the species: 28–33 longitudinal muscle bands (LMBs); short nephridia attached for about a fifth of their length to the body wall; a bilobed brain with short digitate processes; and the origins of the retractor muscles spreading over LMBs 2–5 (ventral retractors) and 9–12 (dorsal retractors). Later, Cutler & Cutler (1985) synonymized *Sipunculus titubans titubans* Selenka & Bülow, 1883, and *Sipunculus titubans diptychus* Fischer, 1895 with *S. nudus*, and broadened the description of the species to encompass the morphology of the synonymized species. The emended morphological diagnosis of *S. nudus* included 24–34 LMBs; the digitate processes on the brain may form a dorsal tuft, which can be divided to varying degrees or can have a sponge-like appearance; the LMBs usually splitting in the glans region (Cutler 1994). Therefore, Cutler & Cutler (1985) extended the definition of the species to accommodate the new morphological variation for the species placed under synonymy. It remains unclear whether this was done due to the presence of a continuity of intermediate states between, *e.g.* the different brain types, or because they thought that these characters bear no taxonomic information.

Sipunculus nudus can be very abundant on the coasts of the Atlantic Ocean and the Mediterranean Sea. For many decades, European scientists used it as a model for physiological and biochemical studies (*e.g.* Andreae 1882). Consequently, *S. nudus* became the most renowned sipunculan species. Today, this worm is popular as fish bait, for example in Spain and is also a source of food in some parts of China and Vietnam; it has therefore become an important fisheries resource – the species has even been overexploited to satisfy the international bait market and food resource demand (Ha *et al.* 2007; Du *et al.* 2009).

However, the existence of so many supposedly widespread sipunculan species remains understudied. Schulze *et al.* (2012) pointed out four alternative hypotheses: (1) efficiency in long-distance dispersal through their long-lived larvae; (2) cryptic speciation; (3) taxonomic lumping, and (4) inadequate morphological information. The first provides a plausible explanation for a true cosmopolitan (or widespread) distribution, but the other three hypotheses suggest that the reported distributions may in fact be artifactual (*e.g.* Kawauchi & Giribet 2010; Schulze *et al.* 2012). The presence of a pelagosphaera larva in *S. nudus* – a long-dispersal teleplanic larval form capable of living in the water column for months and dispersing over long distances (Scheltema & Hall 1975) – could provide a plausible mechanism for its purported cosmopolitan distribution. Nevertheless, the scarcity of diagnostic morphological characters and in some cases the poor definition of these characters, may have led to over-excessive lumping, resulting in its long list of synonyms (see Saiz-Salinas 2013).

Using a multilocus phylogeny of the genus and a careful examination of morphological data, we evaluate the cosmopolitanism of *S. nudus* and present evidence that it consists of a complex of morphospecies. We analysed the morphology of multiple specimens of *S. nudus* collected from 11 localities and performed a phylogenetic analysis using four molecular markers (two mitochondrial and two nuclear). Also, to test the relationship of our samples to those specimens from previous genetic studies (Du *et al.* 2008, 2009), we included the COI and 16S rRNA data from Southern Chinese specimens available in GenBank.

Material and Methods

Thirty-three specimens of *S. nudus sensu lato* from 11 localities (Fig. 2) were used in this study (Table 1). We sequenced up to 3.7 kb of genomic DNA for 25 new specimens. Sequences from eight specimens from previous studies generated in our laboratory (and therefore available for morphological examination), were downloaded from GenBank (Table 2). In addition, we obtained 32 sequences of COI and 16S rRNA available in GenBank (Table 3) for *S. nudus* from Southern China (Fig. 2; Du *et al.* 2008, 2009).

Sample collection, DNA extraction and sequencing

Specimens of *S. nudus* were mostly collected by hand in the intertidal zone at low tide, or at the beach after storms. The Solomon Island and South African samples were dredged from deeper waters (Appendix 1). All specimens were collected and preserved in 96% ethanol except for samples from the Solomon Islands, which were preserved in 70% ethanol (and kept at room temperature). The only sample obtained from South Africa was preserved in RNA*later* QIAGEN© (AMBION, Inc., Austin, TX, USA). Most specimens were stored at −20 °C (recent collections) or −80 °C (older collections) until DNA extraction.

Molecular loci consisted of one nuclear ribosomal gene (28S rRNA, henceforth 28S), one mitochondrial ribosomal gene (16S), one nuclear protein-coding gene (histone H3), and one mitochondrial protein-coding gene (COI). Genomic DNA extraction, amplification, and sequencing were conducted as described by Kawauchi *et al.* (2012). New sequences were deposited in GenBank under accession numbers KF042388–KF42469 for 16S, H3 and COI, and KF110791–KF110798 for 28S (Table 2).

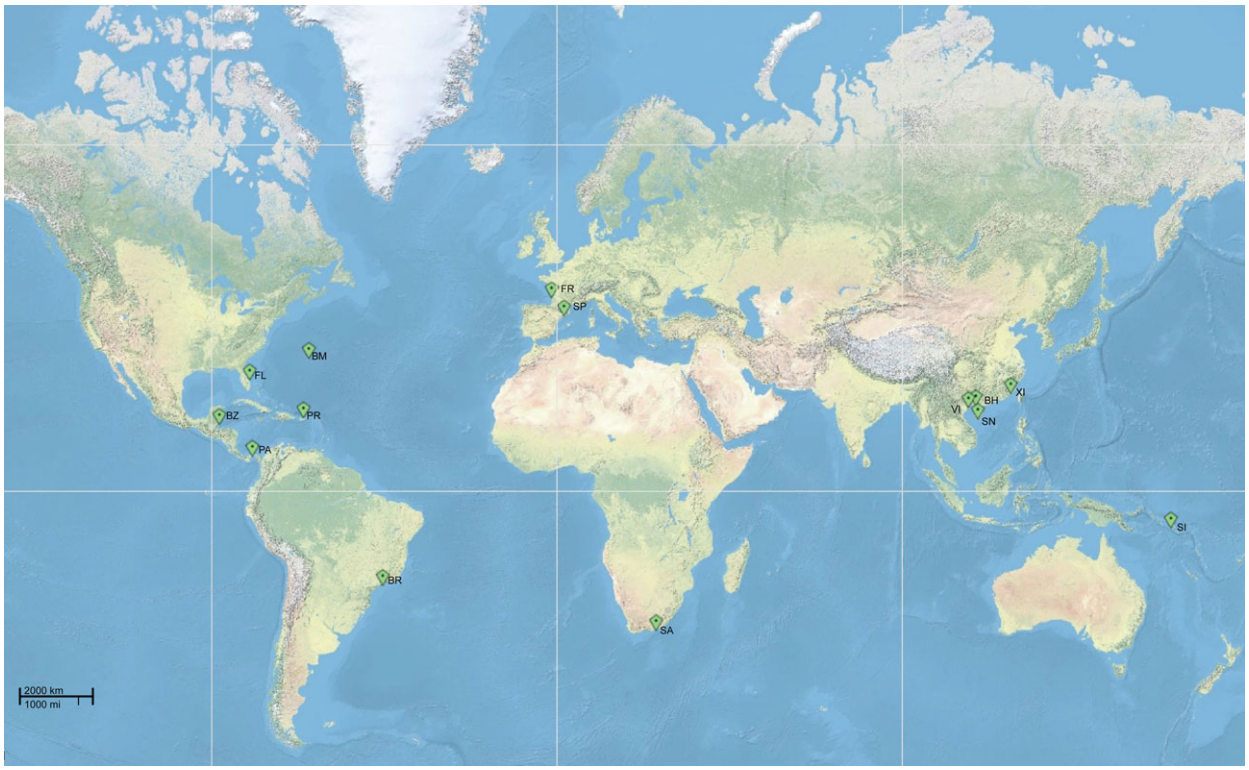

Fig. 2. Sampling localities of *Sipunculus nudus sensu lato*. Localities from specimens collected for this study: Bermuda (BM), Brazil (BR), Belize (BZ), Florida (FL), France (FR), Panama (PA), Puerto Rico (PR), South Africa (SA), the Solomon Islands (SI), and Vietnam (VI). Localities for specimens from Southern China obtained from GenBank: Beihai (BH), Sanya (SN), and Xiamen (XI).

Phylogenetic analyses

Maximum likelihood (ML) and Bayesian inference (BI) analyses were conducted on a static alignment, which was inferred as follows. Outgroup taxa consisted of a selection of genera from three sipunculan families sequenced in our laboratory (Kawauchi *et al.* 2012), and all available diversity in the family Sipunculidae (Table 2). Sequences of the ribosomal genes were aligned using MUSCLE v. 3.6 (Edgar 2004) with default parameters and subsequently treated with GBLOCKS version 0.91b (Castresana 2000) to remove positions of ambiguous homology. The protein-encoding genes COI and histone H3 were also aligned using MUSCLE v. 3.6 with default parameters, and the alignments were confirmed using amino acid sequence translations. No indels were permitted within blocks for any data partition. The size of each gene prior and subsequent to treatment with GBLOCKS is provided in Table 4.

Maximum likelihood (ML) analysis was performed using RAXML v. 7.2.6 (Stamatakis 2006) on 20 CPUs in a cluster at Harvard University, FAS Research Computing (odyssey.fas.harvard.edu). For ML searches, a unique GTR model of sequence evolution with corrections for a discrete gamma distribution (GTR + Γ) was specified for each data partition, as this is the only elaborate model supported in RAXML, and 100 independent searches were conducted. Nodal support was estimated via the rapid bootstrap algorithm (1000 replicates; Stamatakis *et al.* 2008) using the GTR-CAT model. Bootstrap resampling frequencies (BS) were thereafter mapped onto the optimal tree (Fig. 3).

We also performed Bayesian analyses in MRBAYES v. 3.1.2 (Ronquist & Huelsenbeck 2003; Huelsenbeck & Ronquist 2005) with a GTR model of sequence evolution with corrections for a discrete gamma distribution and a proportion of invariant sites (GTR + Γ + I), as selected in JMODELTEST (Darriba *et al.* 2012) under the Akaike information criterion (Posada & Buckley 2004; Table 4). Default priors were used starting with random trees. Two runs, each with three hot and one cold Markov chain, were conducted until the average deviation of split frequencies reached <0.01 (30,000,000 generations). We discarded samples after burn-in, and sampled trees were combined in a majority rule consensus topology. The percentage of nodes was taken as clade posterior probabilities (henceforth PP), and mapped onto the ML optimal tree (Fig. 3).

Table 1. Museum accession numbers (MCZ IZ and DNA numbers), locality, and total number of specimens used for molecular and morphological analyses.

IZ accession number	Locality (n)	DNA number	mol.	mor.
130419	Puerto Rico (1)	DNA100234	✓	✓
130420	France (3)	DNA100245-1	✓	✓
		DNA100245-2	✓	✓
		DNA100245-3	✓	✓
130421	Vietnam (1)	DNA100246-1	✓	✓
130422	Bermuda (1)	DNA100468	✓	✓
130423	Panama (2)	DNA100629-1	✓	✓
		DNA100629-2	✓	✓
130424	Belize (1)	DNA100993	✓	✓
130426	Brazil (1)	DNA101882	✓	✓
130427	France (2)	DNA101884-1	✓	–
		DNA101884-2	✓	–
130428	USA, Florida (1)	DNA102316	✓	–
130430	USA, Florida (6)	DNA103527-1	✓	✓
		DNA103527-2	✓	✓
		DNA103527-3	✓	✓
		DNA103527-4	✓	✓
		DNA103527-5	✓	✓
		DNA103527-6	✓	–
130432	Solomon Islands (7)	DNA103549-1	✓	✓
		DNA103549-2	✓	✓
		DNA103549-3	✓	✓
		DNA103549-4	✓	✓
		DNA103549-6	✓	✓
		DNA103549-7	✓	–
		DNA103549-8	✓	–
130433	Solomon Islands (3)	DNA103550-1	✓	✓
		DNA103550-5	✓	✓
		DNA103550-7	✓	✓
130435	Spain (3)	DNA103730-1	✓	–
		DNA103730-2	✓	✓
		DNA103730-3	✓	✓
130440	South Africa (1)	DNA106941	✓	✓
		Total	33	27

Morphological data

Morphological observations of important diagnostic characters traditionally used to identify species of the genus *Sipunculus* (Cutler & Cutler 1985; Cutler 1994) were conducted in 27 specimens from the 33 used for molecular analysis (Table 1). Specimens were dissected and the following structures were recorded: (1) number of LMBs, counted at the base of the introvert retractors; (2) state of LMBs at the glans region (bifurcated *versus* non bifurcated); (3) degree of nephridia attachment from the body wall (percentage of the length attached to the body wall); (4) state of the origin of the retractor muscles (a separate retractor muscle origin with subdivision of each into a fascicle connected to separate LMBs *versus* a base of the retractor muscle connected by a sheet of muscle; (5) brain shape; (6) shape of the digitate processes – a structure that can be present at the dorsal side of the brain. For the populations with the largest available number of specimens, the Solomon Islands and Florida, eight and five specimens, respectively, were analysed externally and dissected to observe the internal anatomy and identify the specimens to species level. For localities with up to three specimens (Bermuda, Belize, Brazil, Panama, Puerto Rico, South Africa, and Vietnam), all exemplars were dissected. Some specimens had parts of the body missing (Bermuda, Brazil, France, Puerto Rico – one specimen each; and Panama – two specimens). Nevertheless, they were dissected to recover as much information as possible. Table 5 summarizes the morphological observations for these specimens.

Results

Phylogenetic analyses

Our ML analysis recovered monophyly of the family Sipunculidae (*sensu* Kawauchi *et al.* 2012) but a paraphyletic *Sipunculus nudus sensu lato*, due to the placement of *Xenosiphon branchiatus* and *Sipunculus norvegicus* (Fig. 3). Runs of MRBAYES reached stationarity in 5,000,000 generations; 6,000,000 generations were discarded as burn in. The 50% majority rule consensus tree from BI analysis recovered a similar topology. Both sets of analyses resulted into seven major clades in the family Sipunculidae, identified by the Roman numerals I–VII (Fig. 3). The first clade (BS = 100%, PP = 1.00) includes *Sipunculus phalloides* Pallas, 1774, which appears nested within *Sipunculus polymyotus* Fisher 1947; and is the sister group to all the remaining samples. The monophyly of Clades II–VII has low nodal support or posterior probability. Clade II comprises all specimens from the Solomon Islands (BS = 99%, PP = 0.81), which may constitute a new species, and divides into two deep clades. Within Clade III, although without significant support, we find *X. branchiatus* and *S. norvegicus* Danielssen, 1869, and this constitutes the sister clade to all *S. nudus sensu lato* specimens (BS = 68%, PP = 0.92). Although nodal support for each putative species is high, relationships among these clades generally receive low nodal support (Fig. 3). Clade IV includes the two samples from the Pacific side of Panama. Clade V includes all the *S. nudus* from Spain (Mediterranean) and France (Atlantic). Clade VI includes all the samples from Florida, deeply nested within a clade of SW Atlantic and Caribbean exemplars. Finally, our only South African individual forms part of Clade VII as the sister group to two clades of Southern Chinese populations, one from Xiamen and a second

Table 2. GenBank accession numbers for each sequenced fragment. Sequences generated for this study appear in boldface.

MCZ DNA Acc. no.	Species name	28S	COI	16S	H3
100234	*Sipunculus* aff. *nudus*	JN865047	DQ300160	JN864959	JN865127
100468	*Sipunculus* aff. *nudus*	DQ300048	DQ300162	JN864962	DQ300091
100993	*Sipunculus* aff. *nudus*	DQ300049	DQ300164	JN864964	DQ300093
101882	*Sipunculus* aff. *nudus*	JN865051	JN865108	JN865000	JN865128
102316	*Sipunculus* aff. *nudus*	JN865050	JN865107	JN865004	JN865167
103527-1	*Sipunculus* aff. *nudus*	**KF110793**	**KF042451**	**KF042397**	**KF042435**
103527-2	*Sipunculus* aff. *nudus*	–	**KF042452**	**KF042398**	**KF042434**
103527-3	*Sipunculus* aff. *nudus*	–	**KF042453**	**KF042399**	**KF042433**
103527-4	*Sipunculus* aff. *nudus*	–	**KF042454**	**KF042300**	**KF042432**
103527-5	*Sipunculus* aff. *nudus*	**KF110794**	**KF042455**	**KF042301**	**KF042431**
103527-6	*Sipunculus* aff. *nudus*	–	**KF042456**	**KF042302**	**KF042430**
100629-1	*Sipunculus* aff. *nudus*	JN865048	DQ300163	JN864963	DQ300092
100629-2	*Sipunculus* aff. *nudus*	**KF110797**	**KF042448**	**KF042394**	**KF042438**
103549-1	*Sipunculus* sp. 1	–	**KF042457**	**KF042303**	**KF042429**
103549-2	*Sipunculus* sp. 1	–	**KF042458**	**KF042304**	**KF042428**
103549-3	*Sipunculus* sp. 1	–	**KF042459**	**KF042305**	**KF042427**
103549-4	*Sipunculus* sp. 1	–	**KF042460**	**KF042306**	**KF042426**
103549-6	*Sipunculus* sp. 1	–	**KF042461**	**KF042307**	**KF042425**
103549-7	*Sipunculus* sp. 1	–	**KF042462**	**KF042308**	**KF042424**
103549-8	*Sipunculus* sp. 1	–	**KF042463**	**KF042309**	**KF042423**
103550-1	*Sipunculus* sp. 1	–	**KF042464**	**KF042310**	**KF042422**
103550-5	*Sipunculus* sp. 1	**KF110798**	**KF042465**	**KF042311**	**KF042421**
103550-7	*Sipunculus* sp. 1	–	**KF042466**	**KF042312**	**KF042420**
100246-1	*Sipunculus* sp. 2	AF519270	DQ300161	JN864961	AF519295
106941	*Sipunculus* sp. 2	**KF110791**	**KF042445**	–	**KF042316**
100245-1	*Sipunculus nudus*	AF519269	JN865105	JN864960	JN865148
100245-2	*Sipunculus nudus*	–	**KF042446**	**KF42392**	**KF042440**
100245-3	*Sipunculus nudus*	–	**KF042447**	**KF42393**	**KF042439**
101884-1	*Sipunculus nudus*	**KF110795**	**KF042449**	**KF042395**	**KF042437**
101884-2	*Sipunculus nudus*	**KF110796**	**KF042450**	**KF042396**	**KF042436**
103730-1	*Sipunculus nudus*	–	**KF042467**	**KF042313**	**KF042319**
103730-2	*Sipunculus nudus*	**KF110792**	**KF042468**	**KF042314**	**KF042318**
103730-3	*Sipunculus nudus*	–	**KF042469**	**KF042315**	**KF042317**
101069	*Sipunculus novergicus*	JN865046	DQ300159	**KF42391**	DQ300090
104749	*Sipunculus novergicus*	–	**KF042443**	**KF42390**	**KF042442**
101917	*Sipunculus phalloides*	JN865052	DQ300165	JN865003	DQ300094
101121	*Sipunculus polymyotus*	JN865053	DQ300166-1	**KF42389**	DQ300095
105903	*Sipunculus polymyotus*	–	**KF042444**	**KF42388**	**KF042441**
101003	*Golfingia elongata*	JN865057-1	DQ300123-1	JN864985-1	JN865156-1
102604	*Nephasoma pellucidum*	JN865062-1	JN865111-1	JN865008-1	JN865157-1
101080	*Onchnesoma steenstrupii*	DQ300034-2	JN865113-1	–	DQ300074-1
100199	*Phascolopsis gouldii*	AF519272-2	DQ300134-2	JN864952-1	AF519297-1
101006	*Phascolion psammophilus*	DQ300036-2	DQ300133-2	JN864986-1	JN865163-1
100622	*Siphonosoma cumanense*	AY445139	DQ300156	JN864977	AY326296
101084	*Themiste pyroides*	JN865069-1	DQ300171-1	JN864995-1	DQ300098-1
101086	*Xenosiphon branchiatus*	DQ300050	DQ300172	JN864996	DQ300101

clade with individuals from Beihai and Sanya, which also includes our single Vietnam sample.

Morphology

The fixation method used to preserve most of the putative *Sipunculus nudus* specimens for this study was not ideal for morphological analyses. Except for specimens from the Solomon Islands, which were relaxed and fixed in 70% EtOH, the remainder were preserved for molecular techniques (fixed in 96% EtOH or RNA*later* QIAGEN©), which renders the tissue recalcitrant to manipulation and dissection. Consequently, observations of some of the structures were compromised due to the contraction of the

Table 3. GenBank accession numbers for the GenBank sequences from Southern China (Du *et al.* 2008, 2009).

Locality	Species name	COI	16S
Beihai-1	*Sipunculus* sp. 2	FJ788907 1	EU260100 1
Beihai-2	*Sipunculus* sp. 2	FJ788908 1	EU260101 1
Beihai-3	*Sipunculus* sp. 2	FJ788909 1	EU260102 1
Beihai-4	*Sipunculus* sp. 2	FJ788910 1	EU260103 1
Beihai-5	*Sipunculus* sp. 2	FJ788911 1	EU260104 1
Beihai-6	*Sipunculus* sp. 2	FJ788913 1	EU260108 1
Sanya-1	*Sipunculus* sp. 2	FJ788914 1	EU260109 1
Sanya-2	*Sipunculus* sp. 2	FJ788915 1	EU260110 1
Sanya-3	*Sipunculus* sp. 2	FJ788916 1	EU260111 1
Sanya-4	*Sipunculus* sp. 2	FJ788917 1	EU260112 1
Sanya-5	*Sipunculus* sp. 2	FJ788918 1	EU260113 1
Xiamen-1	*Sipunculus* sp. 2	FJ788919 1	EU260114 1
Xiamen-2	*Sipunculus* sp. 2	FJ788920 1	EU260115 1
Xiamen-3	*Sipunculus* sp. 2	FJ788921 1	EU260116 1
Xiamen-4	*Sipunculus* sp. 2	FJ788922 1	EU260117 1
Xiamen-5	*Sipunculus* sp. 2	FJ788913 1	EU260108 1

Table 4. Size of each gene prior and subsequent to treatment with GBLOCKS, best-fit model selected by JMODELTEST (Darriba *et al.* 2012) using the Akaike information criterion (Posada & Buckley 2004), and the model implemented in the MRBAYES analysis.

Partitions	Original length of alignment (bp)	Final length of alignment (bp)	Model selected (AIC)	Model implemented in MRBAYES v.3.1.2
16S rRNA	566	357	TIM2 + I + G	GTR + I + G
28S rRNA	2350	1731	TIM2 + I + G	GTR + I + G
COI	815	814	TPM2uf + I + G	GTR + I + G
histone H3	327	326	GTR + I + G	GTR + I + G

body. Species identifications were confirmed using Cutler's (1994) taxonomic keys, and all exemplars from this study, except for one from the Solomon Islands that has 21 LMBs, fit Cutler's description of *S. nudus*.

Discussion

Sipunculus nudus is not monophyletic

A morphological phylogenetic study of the sipunculan genera previously placed *Xenosiphon* as sister group to *Sipunculus* (Cutler & Gibbs 1985). Recent phylogenetic analyses (Schulze *et al.* 2007; Kawauchi *et al.* 2012) have recovered paraphyly of *Sipunculus*, with respect to *Xenosiphon branchiatus*, somehow supporting Cutler & Gibbs' (1985) hypothesis, which used genera as terminals and hence could not refute generic monophyly. Our analyses corroborate these earlier molecular studies, questioning the validity of the genus *Xenosiphon*. This taxonomic problem will be addressed elsewhere.

Our results, although without strong support, exclude the specimens from the Solomon Islands (Clade II) from the group of other putative *S. nudus* (Fig. 3). Initially, we tentatively identified these specimens as *Sipunculus norvegicus*, due to the presence of a distinct glans region marked off from the trunk by a ridge in some specimens, and the fact that they were dredged from deeper waters (>250 m). However the number of LMBs (25–28) for the majority of the samples (seven specimens) is above the range described for *S. norvegicus* (20–24 LMBs). Two other *Sipunculus* species have LMBs within the range of the Solomon specimens, *Sipunculus longipapillosus* Murina, 1968, from the Red Sea, and *Sipunculus robustus* Keferstein, 1865, from Wallis Is. (currently part of Wallis and Futuna). Nevertheless, we reject the possibility of our specimens being one of these two species for the following reasons: *S. longipapillosus* has elongated papillae in the mid-trunk, which are otherwise observed only in *Xenosiphon branchiatus*, and in *S. robustus* the brain has a lateral, and string-like digitate processes, a condition exclusive to this species. With the exception of one individual with 21 LMBs, which has morphological characters in accordance with *S. norvegicus*, all the others fit the description of *S. nudus sensu* Cutler & Cutler (1985) but differ morphologically from the European specimens studied. Clade II was always recovered as separate from *S. norvegicus* and the other putative *S. nudus* (Fig. 3). Whereas the morphology of the Solomon Islands samples is intermediate between *S. novergicus* and *S. nudus*, the phylogenetic analyses suggest an independent evolutionary history.

In an attempt to compare Solomon Island specimens with synonymized species of *S. nudus*, only *Sipunculus eximioclathratus* Baird, 1868, from the Philippine Islands, was described from a place geographically close to the collection site of our samples. All other synonyms are from the Atlantic Ocean and Mediterranean Sea. The morphology described for *S. eximioclathratus* with 30–33 LMBs and a simple brain, does not correspond with the morphology observed from Solomon specimens, consequently we also discard the possibility of resurrecting *S. eximioclathratus* for these specimens. The number of LMBs, the condition of all LMBs splitting at the glans region, and the absence of a brain digitate process set these samples apart from other putative *S. nudus*, which corroborates the results recovered by the phylogenetic analysis of an independent evolutionary history for Clade II. Therefore, it is possible that Clade II comprises a new species to science, and thus we refer to these specimens as *Sipunculus* sp. 1.

The most salient result of this study is that *S. nudus*, *sensu* Cutler (1994), constitutes four clades that correspond to specific geographic regions. The identification

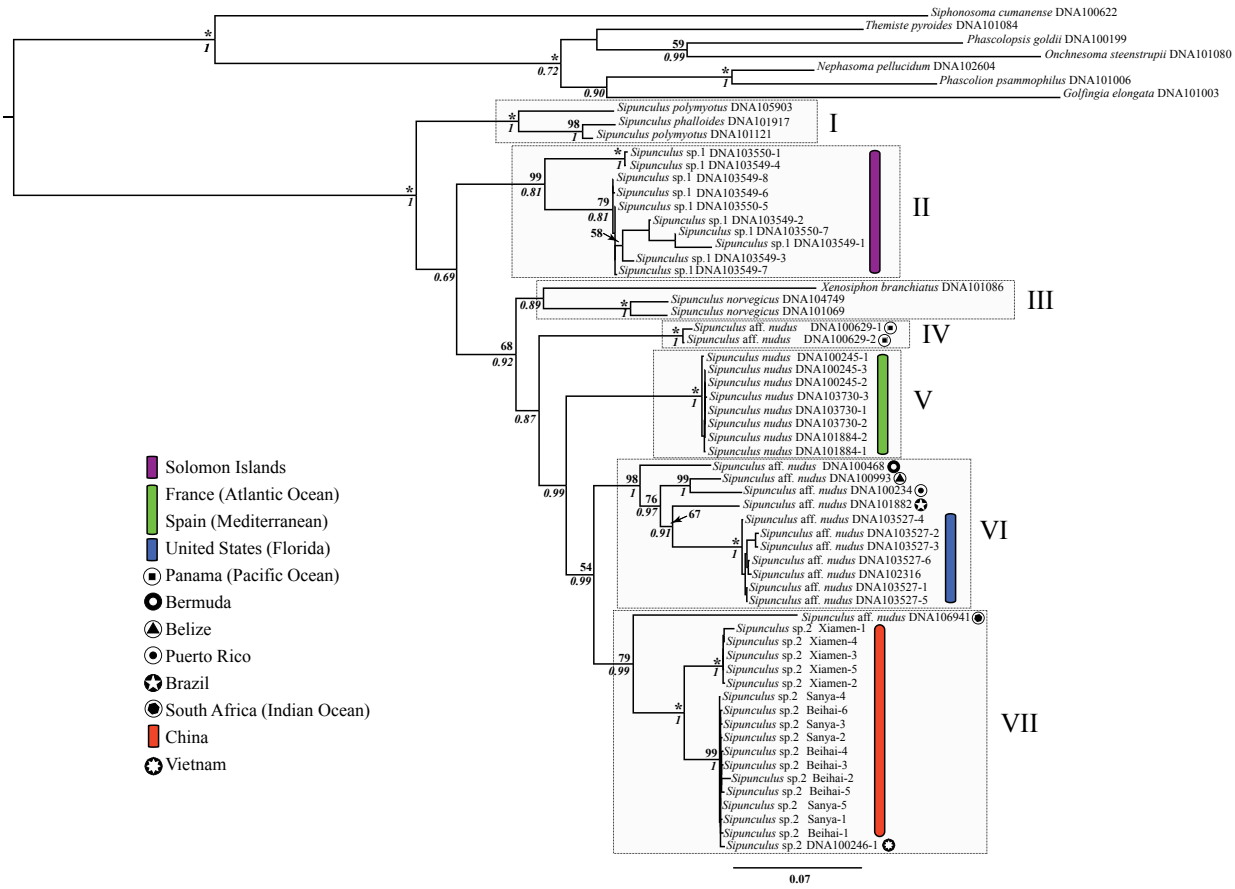

Fig. 3. Optimal maximum likelihood tree (−ln = −21714.009911) for the combined analysis of all data. Numbers above branches indicate bootstrap support values (only BS >50% are indicated); an asterisk indicates a BS value of 100%. Numbers below branches are posterior probability values. Clades are identified by Roman numerals I–VII.

of four additional well-delimited genetic lineages (Clades IV, V, VI, and VII) of *S. nudus*, with nodal support and fixed morphological disparity, thus does not favor the cosmopolitan status of the species. In addition, *p*-distances measured within and among clades for COI show a clear barcoding gap (between 0.00 for Clade V and 0.23 for Clade III *versus* 0.27–0.34 among clades), further supporting our phylogenetic hypothesis of species delimitation (Fig. 3).

Morphological disparity

The number of LMBs is the easiest character to observe and has been broadly used to identify species in the genus *Sipunculus* (Cutler & Cutler 1985). The importance of this character is not unjustified, as observations on the development of the pelagosphera larvae already identified the adult number of LMBs (Hatschek 1883; Fisher 1947; Åkesson 1961). However, a recent study reported that four species with smooth body wall musculature as adults, present circular and sometimes longitudinal body wall musculature splitting into bands during the earlier developmental stages, which will fuse later to form a smooth sheath (Schulze & Rice 2009). That same study reconstructed a body wall musculature arranged in bands as the most likely ancestral character state in sipunculans, reinforcing the importance of the number of LMBs in sipunculan systematics.

The other morphological features used to separate species of *Sipunculus* in Culter's (1994) key are the origin of the retractor muscle, the brain process, and the degree of attachment of the nephridia to the body wall, and they seem useful to separate the five clades in the *S. nudus* complex recovered in our phylogenetic analysis. Indeed, the utility of these morphological characters was well presented in Ditadi's (1982) study of different *Sipunculus* species from Brazilian waters. We also explored the splitting of the LMBs at the glans region, as suggested by Cutler & Cutler (1985), as a possible new character. Our results reinforce the consistent pattern of the status of the

Table 5. Morphological characters investigated in this study. Characters not observed are marked with an asterisk.

			LMBs			Retractor muscle		Brain	
IZ accession number	Locality	DNA number	n	State at glans region	Nephridia attachment	VL/VR	DL/DR	Shape	Digital process
130419	Puerto Rico	DNA100234	28	All split	*	*	*	*	*
130420	France	DNA100245-1	31	*	10%	*/1–8	*/8–16	Bilobed	Fringed
		DNA100245-2	31	Not all split	20%	2–6/*	8–14/*	Bilobed	Fringed
		DNA100245-3	31	Not all split	17%	*/1–6	*/9–16	Bilobed	Fringed
130421	Vietnam	DNA100246-1	30	Do not split	54%	1–7/1–7	*/7–14	Bilobed	Solid tuft
130422	Bermuda	DNA100468	23	Not observed	*	*	*	*	*
130423	Panama	DNA100629-1	32	All split	Free	*	*	*	*
		DNA100629-2	32	All split	Free	*	*/8–10	*	*
130424	Belize	DNA100993	28	All split	*	Membrane	Membrane	Bilobed	Solid tuft
130426	Brazil	DNA101882	29	*	41%	*	*	*	*
130430	USA, Florida	DNA103527-1	30	All split	25%	Membrane	Membrane	Bilobed	Solid tuft
		DNA103527-2	30	All split	39%	Membrane	Membrane	Bilobed	Solid tuft
		DNA103527-3	28	All split	15%	Membrane	Membrane	Bilobed	Solid tuft
		DNA103527-4	30	All split	37%	Membrane	Membrane	Bilobed	Solid tuft
		DNA103527-5	30	All split	43%	Membrane	Membrane	Bilobed	Solid tuft
130432	Solomon Islands	DNA103549-1	26	All split	13%	2–5/1–5	*/8–11	Bilobed	Short
		DNA103549-2	26	All split	Free	3–4/3–4	7–9/7–9	Bilobed	Absent
		DNA103549-3	25	All split	Free	3–4/3–4	*/9–10	Bilobed	Absent
		DNA103549-4	28	All split	Free	3–4/3–4	8–10/9–11	Bilobed	Absent
		DNA103549-6	21	All split	14%	1–5/1–5	7–11/7–11	Bilobed	Absent
130433	Solomon Islands	DNA103550-1	25	All split	Free	1–4/2–6	*/7–10	Bilobed	Absent
		DNA103550-5	25	All split	Free	3–4/3–4	9–10/*	Bilobed	*
		DNA103550-7	26	All split	Free	1–4/1–4	6–10/7–10	Bilobed	Short
130435	Spain	DNA103730-2	31	Not all split	18%	1–7/1–6	*/8–14	*	*
		DNA103730-3	33	Not all split	23%	1–7/1–6	*/9–14	Bilobed	Fringed
130440	South Africa	DNA106941	28	All split	Free	Membrane	Membrane	Bilobed	Solid tuft

LMBs = longitudinal muscle bands; VL/VR = ventral left/ventral right; DL/DR = dorsal left/dorsal right.

LMBs at the glans region within a population observed by the previous authors, but we require further examination of multiple specimens from larger populations to generate enough data to determine the consistency of this putative character.

Sipunculus titubans titubans Selenka & Bülow, 1883 was described from Puntarenas, in the Pacific side of Costa Rica (Tarifeño 1995), but was synonymized with *S. nudus* by Cutler & Cutler (1985). Our phylogenetic analyses place the two specimens from the Pacific side of Panama in a separate clade from the European *S. nudus*, from which they also differ morphologically. Considering the proximity of our collecting site to the type locality of *S. titubans titubans*, we speculated that the latter may constitute a valid species. However, examination of the holotype of *S. titubans titubans* Selenka & Bülow, 1883 (ZMB 1036; Museum für Naturkunde, Berlin, Germany) showed a different number of LMBs (26 *versus* 32 observed in our Panama samples) and differed in nephridial attachment (attached for half of their length in *S. t. titubans versus* free in our Pamana specimens). Until more variation has been examined in the American Pacific clade to assess the degree of variation of these characters, we cannot confirm that our samples belong to *S. titubans titubans*, and thus we refer to our Central American Pacific specimens as *Sipunculus* aff. *nudus*.

Our samples from France were from the same region of provenance of specimens sampled by Stephen & Edmonds (1972). On the other hand, our specimens from Spain are from the coast of the province of Barcelona, located in the Mediterranean Sea. Indeed, both populations have the same morphology, which fits the description given by Stephen & Edmonds (1972) and Cutler (1994). Both morphological and phylogenetic analyses suggest that our European specimens are conspecific, justifying the junior synonymy *Sipunculus tesselatus* Rafinesque, 1814, a species described based on Mediterranean specimens from Naples (Italy). Given that *S. nudus* was originally described from the coasts of Europe, we conclude that these exemplars from the Atlantic in France and the Western Mediterranean in Spain (Clade V) are the true *S. nudus* described by Linnaeus.

Specimens from Florida (part of Clade VI) have three morphological characters that distinguish them from the European *S. nudus* (Clade V) specimens: the origin of the retractor muscles, the brain process, and the splitting of the LMBs at the glans region. The bases of the retractor muscles are not subdivided into fascicles connected to separate LMBs, as observed in the European specimens. Instead, a contiguous sheet of muscle connects the base of the four retractor muscles. Cutler & Cutler (1985) already noticed this character in many specimens from Florida but concluded that this was part of the internal variation of *S. nudus*. We observed a similar character state in Belize and South African specimens, although only one exemplar was observed for each population. The presence of a distinct state for the origin of the retractor bases may indicate that this character could be of good taxonomic value when combined with other traditional characters. For now we consider the presence of a membrane connecting the bases of the retractors a distinctive character supporting the results obtained in the phylogenetic analyses. A solid brain process present in the Florida population is another morphological character distinguishing them from the European *S. nudus*, which have conspicuous digitate brain processes. Also, at the glans region, all the LMBs are split in two in the Florida specimens, whereas not all of them are divided in the European specimens. In our analysis the Florida specimens are always nested within a clade including other Western Atlantic populations, including Bermuda, the Caribbean and Brazilian specimens; furthermore, this clade never appears as the sister group to the European clade, but as the sister group to the Indo–Pacific clade.

The sister group to the Florida population is one exemplar from Brazil, for which the morphology could not be adequately described in this study. Notwithstanding this, Ditadi (1982) described 69 worms for external and six for internal anatomy from the same area as our sample collection, and the internal anatomy description thereof does not fit the description of the Florida specimens. The most noticeable difference between specimens from the two localities is how the retractor muscles attach to the body wall. Whereas in the Florida samples the origin of the retractor muscle forms a muscle sheet connecting all four retractor bases, Ditadi's Brazilian samples have the retractors subdivided into fascicles connected to separate LMBs, similar to the European populations. Another difference observed was the attachment of nephridia to the body wall. The Florida specimens have the nephridia attached for 15–37% of their length to the body wall, starting almost from the anterior tip, whereas Ditadi described a nephridium with an anterior and a posterior lobe (bilobed nephridium), with the attachment to the body starting towards the middle of the nephridium. A similarity between these two populations is the number of LMBs observed: 28–33 for Ditadi's specimens *versus* 28–30 observed in our Florida specimens. Cutler & Cutler (1985) analysed 77 specimens from Florida in which the number of LMBs was within the usual range for *S. nudus sensu* Cutler (between 28–32 LMBs) and close to what Ditadi observed in his specimens. The brain with a bilobed shape and a solid tuft is another similarity between the Florida and Brazilian populations. We could not compare the state of the LMBs at the glans region in our Brazilian specimen, as its posterior end is missing, and Ditadi (1982) did not mention this character.

A Caribbean clade formed by the specimens from Belize and Puerto Rico samples is sister to the Brazilian and Florida populations. For the Belize sample we were able to observe all the morphological features and we conclude that it fits the description provided for the Florida specimens. For the Puerto Rican specimen we recorded only the number of LMBs, which is the same as for the specimens from Belize (28 LMBs). There is little doubt that these two samples correspond to the same species. The most-basal specimen from Clade VI is the one from Bermuda, but the only feature that we could observe was the 23 LMBs, which is the lowest number of LMBs observed in this clade.

To assess the status of the complex of species that constitute the current taxon *S. nudus*, we revisited the synonyms proposed for this species. *Sipunculus titubans diptychus* Fischer, 1895 was described from Accra (Ghana), on the west coast of Africa, but was also recorded from French Guiana (citations in Stephen & Edmonds 1972). The comparison between the syntypes of *S. titubans diptychus* (ZMH V2029; Biozentrum Grindel/Zoological Museum, Hamburg, Germany) and samples from Clade VI showed that we cannot identify specimens from Florida and Belize with this name for two main reasons: the number of LMBs and the origin of the retractor muscles. *Sipunculus titubans diptychus* Fischer, 1895 presents 28–30 LMBs and a separated retractor muscle originating in a fascicle connected to individual LMBS, which contrasts with the 30–33 LMBs and a base of the retractor muscles connected by a sheet of muscle observed in our Florida and Belize specimens. A comparison with the other members of Clade VI (Bermuda, Brazil and Puerto Rico samples) is impaired by the observation of only few morphological characters in these exemplars due to preservation and lack of samples, but the number of LMBs (28–30 LMBs) suggests that we may be dealing with different morphospecies. At this point, considering the strong geographical signal in *Sipunculus*, we have reservations with respect to the presence of the same species on both sides of the Atlantic.

In summary, we can conclude that within Clade VI there is a noticeable morphological variation among the specimens studied, although they form a distinct well-supported clade with respect to the European samples and most probably with respect to *S. titubans diptychus*. We thus refer to these specimens as *Sipunculus* aff. *nudus*, a name we are also adopting temporarily for the South African specimen. However, to better understand the relationship among specimens from Clade VI, a larger collection from each population is desirable to better determine the species delimitations.

The specimen collected in South Africa is morphologically similar to the ones from Florida, barring only the nephridia, which are free from the body wall. However, phylogenetically this sample is part of Clade VII, and is related to other Indo–Pacific specimens from Vietnam and Southern China (Fig. 3). The placement of the Vietnam specimen among samples from Beihai and Sanya in Southern China is not a surprise as these localities are geographically closer to Vietnam than to Xiamen (Fig. 2). We are thus probably dealing with the same morphospecies (we have not been able to examine the morphology of the Chinese specimens). Considering also that the specimen from Vietnam is the only one among all putative *S. nudus* to have about half of the nephridial length attached to the body wall and with the LMBs undivided at the glans region, we assume that this morphospecies is different from the true *S. nudus*. There are two other *Sipunculus* species that present LMBs not bifurcated at the glans region, *Sipunculus lomonossovi* Murina, 1968 and *Sipunculus indicus* Peters, 1850. Nevertheless, *S. lomonossovi* differs from the Vietnam specimens in having a single-lobed brain with no digitate processes, and ecologically it is found only in deep waters (2500–4300 m), instead of the intertidal zone, where samples from Vietnam and Southern China were collected. In the case of *S. indicus* the nephridiopore is located posterior to the anus, a character observed also in *Sipunculus mundanus* Selenka & Bülow, 1883. In fact the relative position of the nephridiopore with respect to the anus is a diagnostic character that divides the genus *Sipunculus* into two subgenera: *Sipunculus* with the nephridiopore anterior to the anus, and *Austrosiphon* with the nephridiopore posterior to the anus. Considering the exclusion of any other names we conclude that the Vietnam and Southern China samples constitute a distinct morphospecies, not previously described. We refer to these specimens as *Sipunculus* sp. 2.

With the analysis of 27 putative *S. nudus* samples we were able to identify morphological variation among populations which correspond to the clades recovered in the phylogenetic analyses. However, a larger number of samples per population would be desirable to delimit the morphological variation among them, as previously done by Ditadi (1982). We hope to be able to do this in the future.

Conclusion

Our data indicate clearly that putative populations of *S. nudus* are genetically different. Furthermore, the use of multiple loci analysed phylogenetically clearly suggests at least four different lineages among what Cutler (1994) referred to as *Sipunculus nudus*. In addition, a fifth lineage from the Solomon Islands (Clade II) is unrelated to the true *S. nudus* described from European waters.

Although morphological characters remain a limitation in sipunculan taxonomy, we were able to reinforce the utility of the diagnostic characters traditionally used in the identification of these organisms, and to corroborate the utility of the state of the LMBs at the glans region, as suggested by Cutler & Cutler (1985). The combination of the characters analysed in this study (number of LMBs, origin of the retractor muscles, shape of the digitate processes of the brain, degree of nephridial attachment to the body wall, and state of LMBs at the glans region), separates satisfactorily the three best-represented clades morphologically (Clades II, V and VI), and our results support the hypothesis of previous taxonomic 'lumping', resulting in a complex of pseudocryptic species.

In this study we also provide evidence that the current taxonomy of *Sipunculus* is in need of revision, and that genetic data provide a complementary framework for establishing such taxonomic revision. This will require examination of the type material of each of the nominal species and their junior synonyms, something beyond the scope of this study, but the morphologies of the members of several of our clades do not correspond to those of other nominal *Sipunculus* species that we were able to examine. We also acknowledge that a larger sample, with specimens properly fixed for each purpose, is necessary for molecular and morphological analyses.

The importance of studying the genetic diversity, and consequently the biodiversity, of the sipunculan fauna is more than a mere taxonomic problem. In places where *S. nudus* is a source of food of high nutritional value, the wild stock has been overexploited to satisfy the market demand (Ha *et al.* 2007; Du *et al.* 2009; Wang *et al.* 2012). Accurate taxonomic identifications are crucial for the implementation of management and conservation plans to avoid decimating species that are new to science, as is the case of the Indo–Pacific commercial *Sipunculus* species.

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Appendix

Collection data for specimens used in this study, in the following format: MCZ IZ accession number, MCZ DNA voucher number, collecting location, depth, collection date (collector)

Golfingia elongata (Keferstein, 1862): MCZ IZ 130302, MCZ DNA101003—Twin Cays, Belize, intertidal zone, Apr. 20, 2003 (M. E. Rice, A. Schulze).

Nephasoma pellucidum (Keferstein, 1865): MCZ IZ 130328, MCZ DNA102604—Récif du Prony, New Caledonia, 22°15′59.9″ S 166°19′37.3″ E, 6 m, Nov. 15, 2007 (G. Kawauchi and C. Tiago).

Onchnesoma steenstrupii Koren & Danielssen, 1875: MCZ IZ 130348, MCZ DNA101080—R/V Oceanus - Station 23, Southern New England, USA, 39°56.172′ N, 69°34.563′ W, June 15, 2003 (A. Schulze).

Phascolopsis gouldii (Portalés, 1851): MCZ IZ 130395, MCZ DNA100199—Woods Hole, Massachusetts, USA, Sept. 30, 1997 (Marine Biological Laboratory specimen supplies).

Phascolion (*Phascolion*) *psammophilus* Rice, 1993: MCZ IZ 130351, MCZ DNA101006—R/V Sunburst cruise 523, Capron Shoals, Florida, USA, Mar. 18, 2003 (A. Schulze).

Siphonosoma cumanense (Keferstein, 1867): MCZ IZ 130402, MCZ DNA100622—Bath, Barbados, intertidal zone, June 24, 2002 (A. Schulze, J.I. Saiz Salinas).

Sipunculus (*Sipunculus*) *norvegicus* Danielssen, 1869: MCZ IZ 130415, MCZ DNA101069—R/V Oceanus, Southern New England, USA, 39°47.230′ N, 70°46.295′ W, June 14, 2003 (A. Schulze); MCZ IZ 130416, MCZ DNA104749—DIVA-Artabria II cruise, Galicia, Spain, 43°4.0614′N, 11°17.1233′W, Oct. 9, 2009 (G. Giribet, J. Troncoso, V. Urgorri, *et al.*).

Sipunculus aff. *nudus*: MCZ IZ 130419, MCZ DNA100234—Station 53F, No Name Cay, Puerto Rico July 27, 1993 (J. Staton and H. Reichardt); MCZ IZ 130422, MCZ DNA100468—South coast of Stock's Harbor, St David's Island, Bermuda, Aug. 7, 2001 (E. Cutler); MCZ IZ 130423, MCZ DNA100629— Isla Taboguilla, off Panama City, Panama, 13 m, June 20, 2002 (T. Nishikawa); MCZ IZ 130424, MCZ DNA100993—Twin Cays, Belize, intertidal zone, Apr. 24, 2003 (M. E. Rice, A. Schulze); MCZ IZ 130426, MCZ DNA101882—Ponta do Araçá, São Sebastião, Brazil, intertidal zone, June 17, 2003 (G. Kawauchi); MCZ IZ 130428, MCZ DNA102316—Fort Pierce, Florida, USA, intertidal zone, Mar. 1, 2006 (G. Kawauchi and A. Schulze); MCZ IZ 130430, MCZ DNA103527—Fort Pierce, Inlet-north side across Guard station, Florida, intertidal zone, USA, Feb. 22, 2008 (M. E. Rice).

Sipunculus (*Sipunculus*) *nudus* Linnaeus, 1766: MCZ IZ 130420, MCZ DNA100245—near Arcachon (Fishermen's locality unspecified) France, Oct. 30, 2000; MCZ IZ 130427, MCZ DNA101884—Costa Aquitania, unspecified locality, France; MCZ IZ 130435, MCZ DNA103730—Cubelles, platja del bunker, Barcelona, Spain, 41°11′53″N, 1°40′22″E, Dec. 29, 2008 (G. Giribet).

Sipunculus sp. 1: MCZ IZ 130432, MCZ DNA103549—BOA 3, Solomon Islands, CP2830, 10°46.46′S, 162°18.73′E, Sept. 21, 2007; MCZ IZ 130433, MCZ DNA103550—BOA 3, Solomon Islands, CP2831, 10°43.57′S, 162°18.30′E, 264-345 m; Sept. 21, 2007.

Sipunculus sp. 2: MCZ IZ 130412, MCZ DNA100246—unspecified locality, Vietnam, Oct. 30, 2000; MCZ IZ 130440, MCZ DNA106941—Algoa Bay, Port Elizabeth, South Africa, 33°58′242″ S, 26°0′270 E, 90 m, April 29, 2012.

Sipunculus (*Sipunculus*) *phalloides* (Pallas, 1774): MCZ IZ 130442, MCZ DNA101917—Ponta do Araçá, São Sebastião, Brazil, 23°49′02″ S, 45°24′19 W, intertidal zone, (G.Y. Kawauchi) [this specimen corresponds to the sequence erroneously published as MCZ DNA101337 by Schulze *et al.* (2007)].

Sipunculus (*Sipunculus*) *polymyotus* Fisher, 1947: MCZ IZ 100444, MCZ DNA101121—Pelican Beach, Belize, Oct. 24, 2002 (D. Felder, R. Robles); MCZ IZ 130445, MCZ DNA105903—Station 88G, Sand flat off Smithsonian Marine Station in Fort Pierce ("Sipunculan Haven"), Florida, USA, intertidal zone, May 25, 2010 (G. Kawauchi).

Themiste (*Themiste*) *pyroides* (Chamberlin, 1920): MCZ IZ 130392, MCZ DNA101084—Whiffen Spit, Vancouver Island, British Columbia, Canada, Sept. 9, 2003 (A. Schulze, M. E. Rice).

Xenosiphon branchiatus (Fischer, 1895): MCZ IZ 130447, MCZ DNA101086—Tamarindo Beach, Costa Rica, Feb. 8, 2003 (R. Quiros).