

Cryptic species of *Notophyllum* (Polychaeta: Phyllodocidae) in Scandinavian waters

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Abstract The phyllodocid polychaete *Notophyllum foliosum* occurs in two colour morphs in Swedish and Norwegian waters, one palish yellow to grey form with black patches that is restricted to deeper waters and often associated with reefs of the deep-water coral *Lophelia pertusa*, and one usually yellow-orange form with black patches and white spots that is usually encountered on more shallow bottoms. We have sampled the two forms from sympatric occurrences in Norway, and the shallow form from the Swedish west coast. Phylogenetic and haplotype analyses based on the mitochondrial cytochrome c oxidase subunit I (*COI*) gene and the nuclear internal transcribed spacer region (*ITS1*-5.8SrDNA-*ITS2*) unequivocally indicate that the two forms represent different species. We apply the name *N. foliosum* (Sars, 1835) to the ‘shallow form’, and propose *N. crypticum* n. sp. for the ‘deep form’. A lectotype is fixed for *N. foliosum*.

Keywords New species · Sibling species · Molecular analysis · *COI* · *ITS1*-5.8SrDNA-*ITS2* · Lectotype

Introduction

The ocean has been suggested to be a breeding ground for cryptic speciation (Bickford et al. 2007; Knowlton 1993,

2000). Discovering and describing this hidden part of biodiversity is one of the major challenges to the taxonomic community in a world facing a biodiversity crisis (Bickford et al. 2007). The present paper is part of a series in which we analyse Scandinavian polychaete species complexes using mitochondrial, nuclear and morphological data in search for cryptic species. We are focusing our research on polychaetes showing some indications of separation, such as differences in colour, behaviour, or distribution at different scales. Here we present the results relating to the phyllodocid polychaete *Notophyllum foliosum* (Sars, 1835), which has a North-East Atlantic distribution from northern Norway in the north to Sicily in the south (Kato and Pleijel 2002; Pleijel 1993).

As taxonomically circumscribed so far, *N. foliosum* exhibits wide variation in coloration (Pleijel 1993) and has been recorded from depths between 10 and 1280 m (Kato and Pleijel 2002). Specimens from shallow water (less than 100 m depth) tend to be yellow to orange (Fig. 5A–C), while specimens from deeper localities (below 100 m) are palish yellow to greyish (Fig. 6A, B). We refer to these two forms as the ‘shallow form’ and the ‘deep form’. Animals of both forms are more or less speckled with patches of darker pigment on the dorsal cirri. In addition, the shallow form also has small white patches on the dorsal cirri that are absent in the deep form. Kato and Pleijel (2002) stated that the deep form has longer antennae, palps and anterior cirri, more elaborate nuchal organs, and larger dorsal cirri. We have found this form exclusively in dead *Lophelia* and *Lophelia* rubble in western Norway, whereas the shallow form is normally found in shell gravel and stones, although it also co-occurs with the deep form on *Lophelia*.

The genus *Notophyllum* Ørsted, 1843 comprises seven species currently considered as valid (Kato and Pleijel

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2002). The animals are found mainly on hard substrates with shells, among barnacles and mussels or in coral rubble, and are distributed in oceans worldwide. Ørsted (1843) erected *Notophyllum* when proposing and describing the species *N. longum* and *N. viride*. About 70 years later, Bergström (1914) designated *Phyllodoce foliosa* Sars, 1835 from the Bergen area (Norway) as the type species of *Notophyllum*. Bergström treated both of Ørsted's species names as junior synonyms of *N. foliosum* (Sars), and this has been followed by subsequent workers. According to Kato and Pleijel (2002: 1148), *N. foliosum* is unique within *Notophyllum* "in the combination of nuchal organs with one to three lobes, dorsally elongated dorsal cirrophores with capillaries, and long proboscis with two or three indistinctly separated rows of rounded papillae on each lateral side." In their morphology-based phylogenetic analysis Kato and Pleijel (2002) presented one feature as apomorphic in *N. foliosum*: the dorsal portion of segment 1 being narrower than segment 3. However, this character was scored with a question mark for *Notophyllum americanum* Verrill, 1885, leaving the possibility that the character state is shared between the two species.

In order to assess whether the differences in coloration and morphology observed in *N. foliosum* are due to intraspecific phenotypic plasticity, or whether the shallow and deep forms represent separate species, we sequenced the mitochondrial cytochrome c oxidase subunit I (*COI*) gene and the nuclear *ITS1*-5.8SrDNA-*ITS2* region from a number of specimens from the Swedish and Norwegian west coasts. Distances between the sampling localities range up to 600 nautical miles. If the two forms are conspecific, specimens from shallow and deep localities from the same area would be expected to be genetically closer to each other than to the same form from another geographic area; on the other hand, if the two forms represent separate species, members of either form should be genetically closer to each other, regardless of geographic origin, than to sympatric members of the respective other form.

Material and methods

Specimens were relaxed with magnesium chloride, preserved in formaldehyde (10%) for a few days, rinsed in fresh water and transferred to 70% alcohol, or preserved directly in 95% alcohol for molecular studies. The shallow form was collected in northern Bohuslän on the Swedish west coast, as well as in the Raunefjord near the Bergen area and in the Trondheimsfjord in Norway; the deep form was collected in the Hjeltefjord near Bergen and in the Trondheimsfjord. The geographic origins of the specimens, GenBank accession numbers, and voucher data are detailed

in Table 1. DNA was extracted using DNAeasy Tissue Kit (Qiagen) following the protocol supplied by the manufacturer. We amplified 658 bp of *COI*, and 1026–1165 bp of *ITS1*-5.8SrDNA-*ITS2* with flanking regions of 18S rDNA and 28S rDNA. We used LCO1490 and HCO2198 (Folmer et al. 1994) for *COI*, and ITS18SFPOLY, ITS5.8SFPOLY, ITS5.8SRPOLY, and ITS28SRPOLY (Nygren et al. 2009) for *ITS1*-5.8SrDNA-*ITS2*. PCR mixtures contained ddH₂O, 1 µl of each primer (10 µM), 2 µl template DNA, and puReTaq Ready-To-Go PCR Beads (Amersham Biosciences) in a mixture totalling 25 µl. The temperature profile was as follows: 96°C/240s—(94°C/30s–48°C/30s–72°C/60s)*45cycles—72°C/480s. PCR products were purified with the E.Z.N.A. Cycle-Pure Kit (Omega Bio-tek). Sequencing was performed at MacroGen Inc. facilities (Seoul, Korea). Overlapping sequence fragments were merged into consensus sequences using SeqMan 4.0 (DNASTar).

For *COI* we used GenBank sequences for the outgroups *Eulalia viridis* (Linnaeus, 1767), *Chaetoparia nilssoni* Malmgren, 1867, *Eumida sanguinea* (Ørsted, 1843), *Phyllodoce groenlandica* Ørsted, 1842, *Eteone picta* Quatrefages, 1866, and *Paranaitis katoi* Nygren et al., 2009. For *ITS1*-5.8SrDNA-*ITS2* we used GenBank sequences for the outgroups *Phyllodoce groenlandica* and *Paranaitis katoi*, whereas new sequences were generated for this study for *Eteone picta* (see Table 1). The reason for this more restrictive set of outgroups is that we could not amplify the *ITS* region of some taxa. As we did not see any indication of large intraindividual variation in *ITS* for specimens that we managed to amplify, we did not pursue cloning of the *ITS* region. Sequences of *COI* were aligned manually. Sequences of the *ITS* region were aligned using Clustal X (Thompson et al. 1997) with three different sets of gap/gap length penalties (20/2, 20/5, 30/8). This was performed in order to identify and exclude alignment-ambiguous sites (Gatesy et al. 1993), but excluding all alignment-variable positions was not an option, as this would have left only the phylogenetically uninformative flanking regions of 18S rDNA and 28S rDNA together with the 5.8S rDNA region. Alignment variability was mostly related to the outgroups. Instead, we analysed all three alignments, as described below, to identify which splits in the tree were supported with different alignment parameters. The alignments were left as they were and were not altered by eye. Alignments are available at TreeBase (<http://www.treebase.org>) under study accession number S2512, matrix accession numbers M4793–M4796.

The nuclear and the mitochondrial datasets were analysed separately. Parsimony analysis was performed using PAUP*4.0b10 (Swofford 2002), with heuristic searches option, tree bisection and reconnection (TBR), and 5000 random additions. All characters were treated as unordered

Table 1 Source and voucher data, including GenBank accession numbers, on sequenced specimens (spm) of *Notophyllum foliosum* (shallow form) and *N. crypticum* n. sp. (deep form)

	Origin/Reference	Depth [m]	Habitat	Voucher ^a	<i>COI</i>	<i>ITS1-5.8S-ITS2</i>
Ingroup						
<i>N. foliosum</i> (spm 1)	Hjeltefjorden, Norway	101–125	<i>Lophelia</i> reef	SMNH 106059 ^b	GQ464333	GQ464347
<i>N. foliosum</i> (spm 2)	Koster area, Sweden	23–70	mixed bottom	SMNH 106060 ^b	GQ464334	GQ464348
<i>N. foliosum</i> (spm 3)	Koster area, Sweden	23–70	mixed bottom	SMNH 106061 ^b	–	GQ464349
<i>N. foliosum</i> (spm 4)	Säcken, Sweden	60–70	near <i>Lophelia</i> reef	SMNH 106062 ^b	GQ464335	GQ464350
<i>N. foliosum</i> (spm 5)	Aust-Agder, Norway	33–36	rocks	SMNH 106063 ^b	–	GQ464351
<i>N. foliosum</i> (spm 6)	Trondheimsfjorden, Norway	30–40	mixed bottom	SMNH 106064 ^b	GQ464336	GQ464352
<i>N. foliosum</i> (spm 7)	Aust-Agder, Norway	33–36	rocks	SMNH 106065 ^b	GQ464337	GQ464353
<i>N. foliosum</i> (spm 8)	Raunefjorden, Bergen, Norway	20–30	shell gravel	SMNH 106066 ^b	GQ464338	GQ464354
<i>N. foliosum</i> (spm 9)	Trondheimsfjorden, Norway	30–40	mixed bottom	SMNH 106067 ^b	GQ464339	GQ464355
<i>N. foliosum</i> (spm 10)	Gullmarsfjorden, Sweden	33–36	gravel, stones	SMNH 106068 ^b	GQ464340	GQ464356
<i>N. crypticum</i> (spm 11)	Trondheimsfjorden, Norway	180–250	<i>Lophelia</i> reef	SMNH T-7809a, b ^c	GQ464341	GQ464357
<i>N. crypticum</i> (spm 12)	Trondheimsfjorden, Norway	180–250	<i>Lophelia</i> reef	SMNH T-7808a, b ^c	–	GQ464358
<i>N. crypticum</i> (spm 13)	Trondheimsfjorden, Norway	180–250	<i>Lophelia</i> reef	SMNH T-7810 ^b	GQ464342	GQ464359
<i>N. crypticum</i> (spm 14)	Trondheimsfjorden, Norway	180–250	<i>Lophelia</i> reef	SMNH T-7811 ^b	GQ464343	GQ464360
<i>N. crypticum</i> (spm 15)	Hjeltefjorden, Norway	101–125	<i>Lophelia</i> reef	SMNH T-7812 ^b	GQ464344	GQ464361
<i>N. crypticum</i> (spm 16)	Hjeltefjorden, Norway	101–125	<i>Lophelia</i> reef	SMNH T-7813a, b ^c	GQ464345	GQ464362
<i>N. crypticum</i> (spm 17)	Trondheimsfjorden, Norway	350	<i>Lophelia</i> reef	SMNH T-7814a, b ^c	GQ464346	GQ464363
Outgroup						
<i>Eulalia viridis</i>	Eklöf et al. (2007)			SMNH 90975	AY996122	–
<i>Chaetoparia nilssoni</i>	Eklöf et al. (2007)			SMNH 90970	AY996125	–
<i>Eumida sanguinea</i>	Eklöf et al. (2007)			SMNH 90977	AY996121	–
<i>Phyllodoce groenlandica</i>	Eklöf et al. (2007)			SMNH 97342	EU431164	EU431125
<i>Eteone picta</i>	Eklöf et al. (2007)			SMNH 90971	AY996124	GQ464364
<i>Paranaitis katoi</i>	Nygren et al. (2009)			SMNH 97333	EU431175	EU431132

^a All vouchers for *N. foliosum* and *N. crypticum* n. sp. are hologenophores (Pleijel et al. 2008).

^b Entire specimen fixed in 95% ethanol.

^c Anterior end fixed in formalin, posterior end in 95% ethanol.

and given equal weight. Jackknife values (Farris et al. 1996) were calculated from 5,000 replicates, with 37.1% character deletion, heuristic search option, TBR, three random additions, and maxtrees set to 300. For Bayesian analysis we used MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003); the best-fit models were selected using the Akaike information criterion (AIC) in MrModeltest (Nylander 2004). For *COI*, we applied a General Time Reversible model with gamma distributed rates across sites (GTR+G) for the first positions, a Felsenstein 81 model with a proportion of the sites invariable (F81+I) for the second positions, and a Hasegawa, Kishino and Yano model with gamma distributed rates across sites (HKY+G) for the third positions. For the *ITS* region we used a GTR+G model for *ITS1*, a GTR with equal base frequencies with a proportion of the sites invariable (SYM+I) for 5.8S rDNA for all three alignments, a GTR+I model for the 20/5 and 20/2 alignments of *ITS2*, and a HKY+G model for the 30/8 alignment of *ITS2*. The short flanking regions of 18S rDNA and 28S

rDNA were not included in the analyses as they did not contain any informative sites. The number of generations was set to two millions with four parallel chains (three hot and one cold), sample frequency to 500, and number of runs to two. One fourth of the samples was discarded as burn-in. Parameters were altered in the proposal mechanisms to acquire a span within 20–60% acceptance rates for the moves in the cold chain of each run (Gelman et al. 1995). Proposal rates were not changed. The tree files were analysed in AWTY (Nylander et al. 2007; Wilgenbusch et al. 2004) to assess visually whether the analyses had reached the stationary phase.

The number of haplotypes was determined with the software program TCS 1.21 (Clement et al. 2000). *ITS1* and *ITS2* were analysed separately; boundaries were determined after Chen et al. (2002). When establishing the number of haplotypes we treated gaps as a fifth state as we consider haplotypes separated by an indel event to be different. Statistical parsimony haplotype networks were also generated in TCS 1.21, treating gaps as missing data or as a fifth

state, initially with a 95% connection limit. The reason for this is that there is no obvious way to treat indel events, as the latter may occur as a single inserted/deleted base or as several adjacent inserted/deleted bases. Thus, we wanted to explore both extremes, i.e. either treat indels as missing data or every single inserted/deleted base as a single mutational event, knowing that the truth probably lies somewhere in between. Haplotypes of *N. foliosum* sometimes were too distant for connection in a network, in which cases we also used a fixed connection limit. We only present the haplotype networks from the alignment with gap/gap length penalties 20/5, as the results were similar for all three alignments. Genetic distances were calculated in PAUP*4.0b10. We used the same models selected for *ITS1* and *ITS2* in the Bayesian analysis, but since a codon-specific model may not be specified in PAUP, a new best-fit model, GTR+I, was selected for *COI*, using MrModeltest based on all positions. For the various genes and alignments, number of haplotypes, variation within either form, and distance between forms are summarized in Table 2. Abbreviations used in the text: FP=specimens in the collection of the third author; NHMUO=Natural History Museum, University of Oslo (Norway); SMNH=Swedish Museum of Natural History, Stockholm; spm(s)=specimen(s); ZMUC=Zoological Museum, University of Copenhagen (Denmark).

Results

The *COI* dataset consists of 658 characters, of which 159 are parsimony-informative and 68 variable but not

parsimony-informative. These two groups combined (227 characters) include 48 characters occurring in the first position, 5 in the second, and 174 in the third position. Excluding outgroups, 68 characters are parsimony-informative, and 10 variable but not parsimony-informative. Of the combined 78 characters, 4 occur in the first position, 1 in the second, and 73 in the third position. The tree topologies from the Bayesian and the parsimony jackknife analyses are identical, except that the former is slightly more resolved (Fig. 1, right tree). The results show two distinct and well-supported clades for the ingroup taxa, representing members of the shallow and the deep form, respectively. The mean distance between these two clades is $8.5 \pm 0.95\%$; none of the separating nucleotide substitutions results in amino acid changes. The variation is $1.1 \pm 0.69\%$ within the shallow form, $3.3 \pm 1.7\%$ within the deep form. Seven haplotypes are found in the shallow form; six of them are singletons, one is shared by two specimens. A single haplotype network is formed when applying the 95% connection limit (Fig. 2A). Six haplotypes are found in the deep form; all are singletons. Two separate networks with two and three haplotypes are formed, and one haplotype is single, when applying the 95% connection limit (Fig. 2B).

Depending on the alignment, the *ITS1*-5.8S rDNA-*ITS2* with flanking regions consists of 1320–1369 characters, where 18SrDNA constitutes 35 characters, *ITS1* 698–731 characters, 5.8S rDNA 154 characters, *ITS2* 410–426 characters, and 28SrDNA 23 characters. Of those, 264–342 are parsimony-informative, and 464–497 are variable but not parsimony-informative. Excluding outgroups, 58–

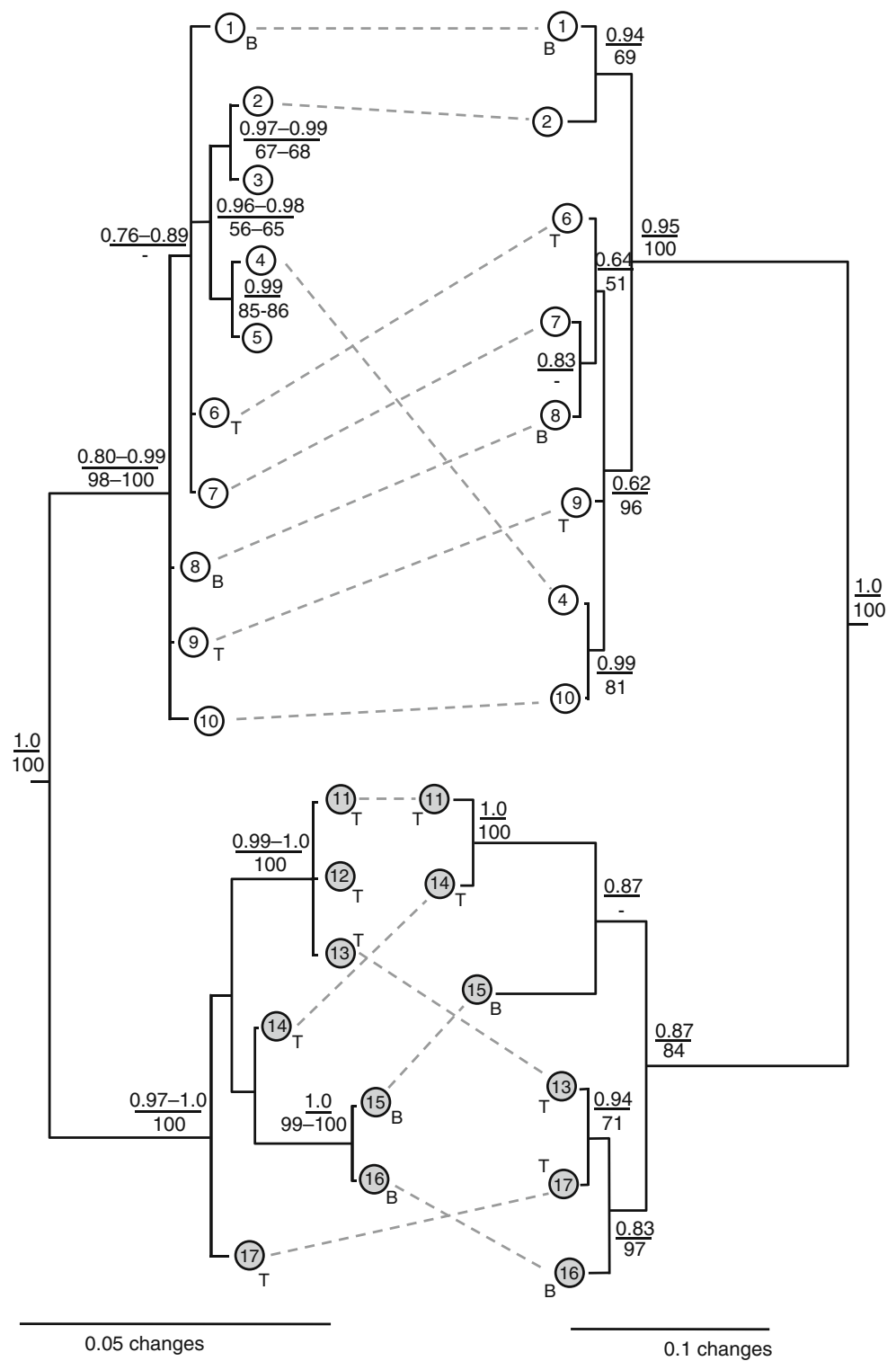
Table 2 Intraspecific variation and interspecific distances concerning the mitochondrial gene *COI* and the nuclear genes *ITS1* and *ITS2* of *Notophyllum foliosum* (shallow form) and *N. crypticum* n. sp. (deep form)

	Gene	No. of specimens	No. of haplotypes	Model ^a	Intraspecific variation [%] ^b	Interspecific distance [%] ^b
<i>N. foliosum</i>	<i>COI</i>	8	7	GTR+I	1.1 ± 0.69	8.5 ± 0.95
<i>N. crypticum</i> n. sp.	<i>COI</i>	6	6	GTR+I	3.3 ± 1.7	
<i>N. foliosum</i>	<i>ITS1</i>	10	8	GTR+G	0.36 ± 0.27	4.9 ± 0.38
					0.36 ± 0.27	4.9 ± 0.38
					0.36 ± 0.27	5.5 ± 0.32
<i>N. crypticum</i> n. sp.	<i>ITS1</i>	7	5	GTR+G	0.83 ± 0.60	
					0.83 ± 0.60	
					1.1 ± 0.97	
<i>N. foliosum</i>	<i>ITS2</i>	10	9	GTR+I/	0.47 ± 0.32	6.3 ± 0.97
				GTR+I/	0.47 ± 0.32	5.4 ± 0.27
				HKY+G	0.47 ± 0.32	11.3 ± 0.69
<i>N. crypticum</i> n. sp.	<i>ITS2</i>	7	4	GTR+I/	2.4 ± 2.3	
				GTR+I/	1.6 ± 1.4	
				HKY+G	2.4 ± 2.3	

^a Selected as best-fit models in MrModeltest 1.21, using the Akaike information criterion.

^b *ITS1* and *ITS2* variation and distances specified for all three alignments; gap/gap length penalties 20/5, 20/2 and 30/8, respectively.

Fig. 1 Majority rule consensus tree from Bayesian analyses of the nuclear *ITS1*–*5.8SrDNA*–*ITS2* data set (*left tree*) and the mitochondrial *COI* data set (*right tree*). Outgroups not shown. Support values at nodes: Bayesian posterior probability (*above line*), parsimony jackknife value (*below line*). *ITS* tree: support values (or ranges) shown only at nodes supported in all three alignments. *White circles*: specimens of *Notophyllum foliosum* (shallow form), *grey circles*: *N. crypticum* n. sp. (deep form); *numbers in circles* correspond to specimen numbers in Table 1 and the “*Material examined*” sections. B=Bergen; T=Trondheimsfjord



81 characters are parsimony-informative, and 7–15 are variable but not parsimony-informative. The resulting tree topologies are largely concordant in all three alignments in both Bayesian and parsimony jackknife analyses, and, similar to the analysis based on *COI*, reveal two non-overlapping clades with high support among the ingroup taxa, representing the shallow and the deep form (Fig. 1,

left tree). The Bayesian analyses are slightly more resolved, and depending on alignment and type of method used, specimen 17 (spm 17) of the deep form alternates with a clade consisting of spms 15 and 16 in the most basal position. Concerning *ITS1*, the mean distance between the shallow and the deep form is 4.9 ± 0.38 – $5.5 \pm 0.32\%$; the variation is $0.36 \pm 0.27\%$ within the shallow form, and $0.83 \pm$

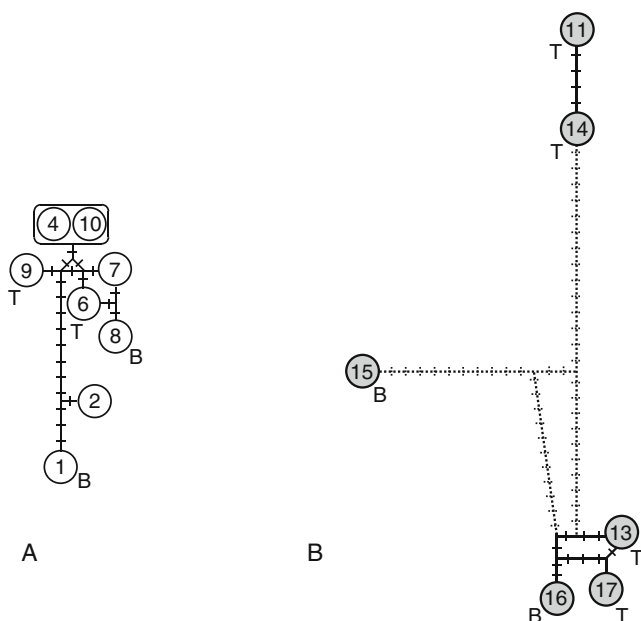


Fig. 2 Haplotype networks for *COI*. (A) *Notophyllum foliosum*. (B) *N. crypticum* n. sp. Each bar across a line connecting two specimens denotes one mutation; shaded lines indicate that a fixed connection limit was necessary to connect all haplotypes. For further explanations, see Figure 1

0.60–1.1±0.97% within the deep form. Eight haplotypes are found in the shallow form; seven are singletons, one is shared by three specimens (Fig. 3B). Single haplotype networks are formed when applying the 95% connection limit, regardless of whether gaps are treated as missing data (Fig. 3A) or as a fifth state (Fig. 3B). Five haplotypes are found in the deep form; three are singletons, two are shared by two specimens each (Fig. 3D). A single haplotype network is formed when applying the 95% connection limit when gaps are treated as missing data (Fig. 3C); two haplotype networks are formed when gaps are treated as a fifth state (Fig. 3D). Concerning *ITS2*, the mean distance between the shallow and the deep

form is 5.4 ± 0.27 – $11.3 \pm 0.69\%$; the variation is $0.47 \pm 0.32\%$ within the shallow form, and 1.6 ± 1.4 – $2.4 \pm 2.3\%$ within the deep form. Nine haplotypes are found in the shallow form; eight are singletons, one is shared by two specimens (Fig. 4B). Single haplotype networks are formed when applying the 95% connection limit, regardless of whether gaps are treated as missing data (Fig. 4A) or as a fifth state (Fig. 4B). Four haplotypes are found in the deep form; two are singletons, one is shared by two specimens, and one is shared by three specimens (Fig. 4D). Two haplotype networks are formed when applying the 95% connection limit, regardless of whether gaps are treated as missing data (Fig. 4C) or as a fifth state (Fig. 4D).

Discussion

The notion of sibling or cryptic species has been around since pre-Linnean times (Winker 2005), and Mayr (1963) used it when arguing in favour of his biological species concept, in contrast to the morphological species concept that prevailed at the time. However, only in the last 20 years have molecular data made it possible to efficiently analyse gene flow and assess how common cryptic species actually are (Saez and Lozano 2005). The marine realm has been suggested as one great hotspot for sibling species (Bickford et al. 2007; Knowlton 1993, 2000). There are several reasons for this. First, our limited access to the ocean environment restricts our knowledge of the appearance of live animals and the specific habitats in which they live, which can mislead us in attempts to assess species boundaries (Knowlton 1993). As has been pointed out before, in most cases marine taxonomists are not as familiar with their study organisms as, for example, ornithologists are with their birds. Second, many marine organisms make use of chemical recognition systems for mate-finding and gamete recognition (Palumbi 1994). Naturally, then, two

Fig. 3 Haplotype networks for *ITS1*. (A, B) *Notophyllum foliosum*. (C, D) *N. crypticum* n. sp. Gaps treated as missing data (A, C) or as a fifth state (B, D). Each bar across a line connecting two specimens denotes one mutation; shaded lines indicate that a fixed connection limit was necessary to connect all haplotypes. For further explanations, see Figure 1

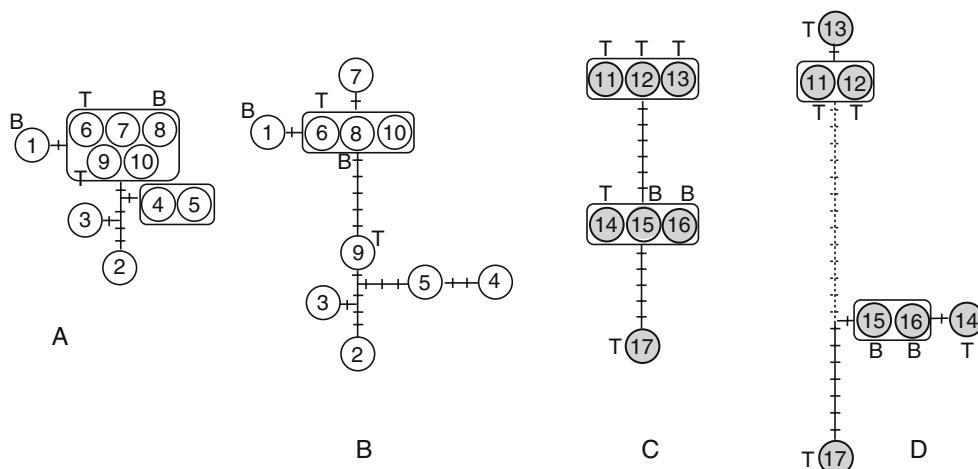
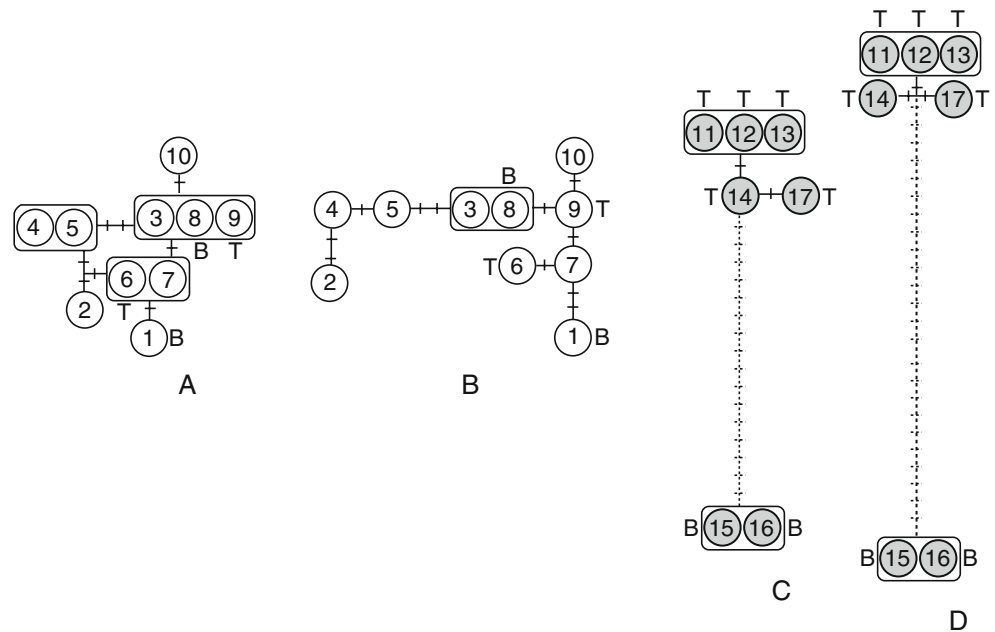


Fig. 4 Haplotype networks for *ITS2*. (A, B) *Notophyllum foliosum*. (C, D) *N. crypticum* n. sp. For further explanations, see Figure 3



species can be perceived as completely different by their respective members, but look similar to us when we examine them visually. Third, many marine taxonomists have worked in an over-conservative tradition, in which large intraspecific variation and large distribution areas have been the norm (Klautau et al. 1999). The present study is the third in a series in which we analyse putative cryptic polychaete species complexes in Scandinavian waters. In the second of these papers (Nygren et al. 2009) we analysed a species group with a wide geographical distribution and differences in maximal size; in the first (Nygren et al. 2005) and the present paper we analyse species groups with variation in colour and habitat preferences.

The results from our phylogenetic analyses confirm that the deep and the shallow form of *N. foliosum* represent two genetically distinct species. Both mitochondrial and nuclear data strongly support two non-overlapping monophyletic groups, corresponding to one deep and one shallow form. The two forms can also be found sympatrically, which provides direct evidence that they are reproductively isolated, indeed (Knowlton and Weigt 1998). Thus, the shallow and the deep form represent separate species both under a phylogenetic (Mishler and Theriot 2000) and a biological (reproductive) species concept (Mayr 1963). It is worth pointing out that concerning ingroup taxa, the trees based on the mitochondrial *COI* and the nuclear *ITS* region only have two nodes in common: those for a shallow and a deep form. Within these two clades, the two genes yield contradictory topologies; e.g. specimens (spms) 4 and 10 of the shallow form (*N. foliosum*) make up a clade with high support in the *COI* tree, but not in the *ITS* tree, and the same is true for spms 13 and 17 of the deep form (*N. foliosum* n. sp.) (Fig. 1). This is not surprising given the

difference in inheritance pattern between the maternally transmitted mitochondria and the recombining, biparentally transmitted nuclear genes (Avice 2000). Notably, the deep form exhibits large intraspecific variation. The reason for this is not understood, and the sample size is not large enough to draw any conclusions. The large intraspecific variation in the deep form is also obvious in the TCS analyses, where the haplotypes always form single networks in the shallow form (Figs. 2A, 3A, B, 4A, B) but less so in the deep form (Figs. 2B, 3D, 4C, D). Hart and Sunday (2007) proposed that the 95% connection limit in statistical parsimony networks could be used to identify species boundaries, but they also pointed out that this method might not be appropriate when analysing small samples, in which case there is a risk of obtaining false positives. Another reason for disconnected networks is cryptic speciation, and this was also our initial thought when looking at the *COI* data alone. Analysis in combination with the nuclear data, however, showed that the large variation in *COI* in the deep form (*N. foliosum*) represents intraspecific variation, since the specimens with the most divergent *COI* haplotypes, i.e. spms 11 and 13, and spms 15 and 16, are closest together in the *ITS* tree. Had we used a simplistic one-gene approach to this specific problem, we would have risked coming to the conclusion that the deep form comprises several different species.

We have found that the two species can be separated morphologically on colouration, with specimens of the deep form palish yellow to grey, those of the shallow form typically yellow-orange. We have also noticed that members of the shallow form always have white spots accompanying the black spots on the dorsal cirri (Fig. 5A), and that these white spots are never present in the deep form. Kato and Pleijel

Fig. 5 Photographs of colour forms of *Notophyllum foliosum*. Note characteristic white spots on dorsal cirri (absent in *N. crypticum* n. sp.). **(A)** Spm 4. **(B)** Unsequenced, unnumbered specimen. **(C)** Spm 1. Scale bars: A=1 mm; B=2 mm; C=2.5 mm



(2002) claimed additional morphological differences between the shallow and the deep form, including in the development of nuchal lobes, length of palps, antennae and dorsal cirri, but we have been unable to confirm consistent differences in these features. All specimens preserved in formalin lose their original colouration and become dark brown, but specimens conserved directly in alcohol keep the white spots characteristic for the shallow type.

Our study underscores the importance of looking at mitochondrial and nuclear as well as morphological data when assessing species boundaries. Without a combined nuclear and mitochondrial approach we would have been misled in a number of cases, and without a thorough morphological investigation in parallel to the molecular findings our newly discovered cryptic species complex would still have been cryptic even after its discovery (Schlick-Steiner et al. 2007).

We conclude that *Notophyllum foliosum* in Scandinavian waters, in the wide sense previously conceived, includes

two species: *N. foliosum*, which occurs in shallow water (at 20–125 m depth) and is characterised by white spots on the dorsal cirri; and *N. crypticum* n. sp., which occurs in deeper waters (101–350 m). For descriptions and nomenclature, see the [Taxonomic section](#) below.

Kato and Pleijel (2002) recorded *N. foliosum* from both the Mediterranean and the North Atlantic coasts of Europe, at depths of 10–1280 m. Due to limited access to specimens preserved for molecular study, the present study focuses on specimens from the Swedish and Norwegian west coasts. We conclude that two distinct species are present in this area, but cannot exclude the possibility that further species exist in the geographic range of the former *N. foliosum* sensu lato, i.e. that the situation is more complicated than presented here. Although we provide a redescription of *N. foliosum* (Sars) and introduce a second, new species, we suggest that the term “*N. foliosum* group” be used whenever there are uncertainties involved.

Taxonomic section

Notophyllum foliosum (Sars, 1835)

(Fig. 5A–C)

Phyllodoce foliosa M. Sars, 1835—Sars (1835: 60–61; pl. 9, fig. 26A–E).

Notophyllum longum Ørsted, 1843—Ørsted (1843: 26; pl. 5, fig. 78).

Notophyllum viride Ørsted, 1843—Ørsted (1843: 26; pl. 5, fig. 87).

Notophyllum foliosum (Sars)—Pleijel and Dales (1991: 126–127, fig. 42); Pleijel (1993: 9–12, figs. 2, 3), in part; Kato and Pleijel (2002: 1145–1150, figs. 1, 3–5), in part.

Material examined

Norway Lectotype (NHMUO C3366-1) and three paralectotypes (NHMUO C3366-2, C3366-3, C3366-4), so designated below (see **Remarks**): Manger, Bergen area. 1 spm (SMNH 106059, fixed in 95% ethanol)=spm 1 in present study: Hjeltefjord, Føllese, 60°24.825'–60°24.667'N, 05°08.478'–05°08.493'E, 125–101 m, rectangular dredge, *Lophelia* reef, coll. FP, 9 Mar 2006. 1 spm (SMNH 106063, fixed in 95% ethanol) = spm 5: Aust-Agder, off Arendal, 58°27.291'–58°27.562'N, 08°55.562'–08°55.373'E, 69–40 m, dredge, rocks, coll. FP, 26 Jun 2006. 1 spm (SMNH 106065, fixed in 95% ethanol)=spm 7: Aust-Agder, off Arendal, 58°27.291'–58°27.562'N, 08°55.562'–08°55.373'E, 69–40 m, dredge, rocks, coll. FP, 26 Jun 2006. 1 spm (SMNH 106064, fixed in 95% ethanol)=spm 6: Trondheimsfjord, Tautra, 63°35.14'N, 10°38.87'E, 30–40 m, triangular dredge, mixed bottom, coll. FP, 12 Jan 2002. 1 spm (SMNH 106066, fixed in 95% ethanol)=spm 8: Raunefjord, Flatenossen, 60°15.999'–60°16.134'N, 05°12.824'–05°12.561'E, 20–30 m, dredge, shell gravel, coll. FP, 16 Nov 2005. 1 spm (SMNH 106067, fixed in 95% ethanol)=spm 9: Trondheimsfjord, Tautra, 63°35.14'N, 10°38.87'E, 30–40 m, triangular dredge, coll. FP, 12 Jan 2002.

Sweden 8 spms (FP): Koster area, Bohuslän. 11 spms (SMNH 22539): Väderöarna, Bohuslän, 40 m, coll. FP, 10 Apr 1990. 3 spms (SMNH 23278): between Vattenholmarna, Koster area, Bohuslän, c. 30–40 m, coll. FP, 23 Apr 1985. 1 spm (SMNH 23279): Koster area, Bohuslän, coll. FP, 25 May 1988. 1 spm (SMNH 23280): Kosterfjord, Bohuslän, coll. FP, 26 Jul 1988. 7 spms (SMNH 23281): E Vattenholmen, Bohuslän, 30 m, coll. FP, 7 Oct 1985. 3 spms (SMNH 23282): Koster area, Bohuslän, coll. FP, 18 Oct 1990. 1 spm (SMNH 23283): Styrsöklåvet, Koster area, Bohuslän, 58°54'N, 11°07'E, coll. FP, 14 Jun 1965. 1 spm (SMNH 106060, fixed in 95% ethanol)=spm 2: Bohuslän, Koster area, Yttre Vattenholmen, 58°52.433'–58°52.486'N, 11°06.240'–11°06.456'E, 70–23 m, rectangular dredge,

mixed bottom, coll. FP, 20 Jul 2005. 1 spm (SMNH 106061, fixed in 95% ethanol)=spm 3: Bohuslän, Koster area, Yttre Vattenholmen, 58°52.433'–58°52.486'N, 11°06.240'–11°06.456'E, 70–23 m, rectangular dredge, mixed bottom, coll. FP, 20 Jul 2005. 1 spm (SMNH 106062, fixed in 95% ethanol)=spm 4: Bohuslän, Säcken, 59°00.842'N, 11°06.978'E, 60–70 m, settling plates near reef of *Lophelia pertusa*, coll. SS, Sep 2006. 1 spm (SMNH 106068, fixed in 95% ethanol)=spm 10: Gullmarsfjorden, 58°13.584'N, 11°24.416'E, 33–36 m, dredge, gravel and stones, coll. FP, 31 Mar 2003.

Description

Kato and Pleijel's (2002) description of *N. foliosum* corresponds to the species as delineated here, including their figs 1A–F, 3A, and 4I. Morphologically, we can only separate *N. foliosum* from *N. crypticum* n. sp. by the colour (see below).

The lectotype designated here is an entire specimen 23 mm in length at 80 segments.

Colour The most common colouration is yellow-orange (Fig. 5C), but dirty white to grey (Fig. 5A) and reddish (Fig. 5B) variants also occur. In addition, the colour can vary along the specimen's body (e.g. Fig. 5B). Eyes dark red to brown. Dorsal cirri speckled with dark pigment and, invariably, with smaller white spots (Fig. 5A–C).

Habitat

Mixed bottoms with sand, shell gravel and stones; occasionally also reefs of *Lophelia pertusa*. Known depth range 20–125 m.

Distribution

Currently known from Denmark, western Norway and the Swedish west coast; further distribution uncertain.

Remarks

Sars' (1835) original description of *N. foliosum* was based on specimens from the area near Bergen in western Norway, but provides no further information on the habitat or depth. However, Sars did mention the presence of white spots on the dorsal cirri, which supports that his description referred to the 'shallow form' in the sense of the present work. Sars' type material, located in the Natural History Museum in Oslo, consisted of four syntypes, all in good condition. The white pigmentation is still clearly visible on

only one of these specimens; we designate this specimen as the lectotype of *Phyllodoce foliosa* M. Sars, 1835. The paralectotypes may or may not be conspecific.

According to our findings, the descriptions under the name *N. foliosum* by Pleijel and Dales (1991), Pleijel (1993), and Kato and Pleijel (2002) were based on (minimally) two different species: *N. foliosum* (Sars) as restricted here, and *N. crypticum* n. sp. We agree with those authors' synonymy statements concerning *N. foliosum* and Ørsted's (1843) two species, *N. longum* and *N. viride*. The latter were described from shallow waters in Denmark; no other species is currently known from this well-studied area. Concerning *N. polynoides* Ørsted, 1845, however, which was described from Drøbak in Norway, we are unable to decide whether it is conspecific with *N. foliosum* or with the new species described below. We consider as even more questionable any possible synonymy with *N. alatum* Langerhans, 1880, *N. frontale* Langerhans, 1880, or *Eulalia oblecta* Ehlers, 1864, because these names were proposed for material from Madeira and the Mediterranean. *Trachelophyllum luetkeni* (Levinsen, 1883), described from near Bergen in western Norway and also synonymised with *N. foliosum* by Pleijel and Dales (1991), Pleijel (1993) and Kato and Pleijel (2002), is discussed in the **Remarks** section on the new species described below.

Notophyllum crypticum n. sp.

(Fig. 6)

Notophyllum foliosum (Sars)—Pleijel and Dales (1991: 126–127, fig. 42), in part; Pleijel (1993: 9–12, figs. 2–3), in part; Kato and Pleijel (2002: 1145–1150, figs. 3–5), in part.

Etymology

The species epithet refers to the previously hidden identity of the species. It is to be treated as adjectival for the purposes of nomenclature.

Material examined

Norway Holotype (SMNH T-7808a=anterior end, fixed in formalin; SMNH T-7808b=posterior end, fixed in 95% ethanol)=spm 12 in present study: Trondheimsfjord, Rødberg, 63°28.093'–63°28.329'N, 09°59.990'–09°59.982'E, 180–250 m, triangular dredge, *Lophelia* reef, coll. FP, 5 Dec 2006. 1 paratype (SMNH T-7809a=anterior end, fixed in formalin; SMNH T-7809b=posterior end, fixed in 95% ethanol)=spm 11: Trondheimsfjord, Rødberg, 63°28.36'N, 10°00.04'E, 180–250 m, triangular dredge, *Lophelia* reef, 29 Jan 2002, coll. FP. 1 paratype (SMNH T-7810, fixed in 95% ethanol)=spm 13: Trondheimsfjord, Rødberg, 63°28.36' N, 09°59.990'–09°59.982'E, c. 250 m, triangular dredge, *Lophelia* reef, 29 Jan 2002, coll. FP. 1 paratype



Fig. 6 Photomicrographs of *Notophyllum crypticum* n. sp., holotype. (A) Entire specimen, dorsal view; scale bar: 4 mm. (B) Close-up of anterior end, dorsal view

(SMNH T-7811, fixed in 95% ethanol)=spm 14: Trondheimsfjord, Rødberg, 63°28.36'N, 10°00.04'E, 180–250 m, triangular dredge, *Lophelia* reef, 21 Feb 2003, coll. FP. 1 paratype (SMNH T-7812, fixed in 95% ethanol)=spm 15: Hjeltefjord, Føllese, 60°24.825'–60°24.667'N, 05°08.478'–05°08.493'E, 125–101 m, rectangular dredge, *Lophelia* reef, 9 Mar 2006, coll. FP. 1 paratype (SMNH T-7813a=anterior end, fixed in formalin; SMNH T-7813b=posterior end, fixed in 95% ethanol)=spm 16: Hjeltefjord, Føllese, 60°24.825'–60°24.667'N, 05°08.478'–05°08.493'E, 125–101 m, rectangular dredge, *Lophelia* reef, 9 Mar 2006, coll. FP. 1 paratype (SMNH T-7814a=anterior end, fixed in formalin; SMNH T-7814b=posterior end, fixed in 95% ethanol)=spm 17: Trondheimsfjord, Rødberg, 63°28.093'–63°28.329'N, 09°59.990'–09°59.982'E, c. 350 m, triangular dredge, *Lophelia* reef, 7 Dec 2006, coll. FP. 1 paratype (SMNH T-7815a=anterior end, preserved in formalin; SMNH T-7815b=posterior end, preserved in 95% ethanol): Trondheimsfjord, Rødberg, 63°28.458'–63°28.508'N, 10°00.186'–10°00.192'E, 200–187 m, triangular dredge, *Lophelia* reef, 7 May 2009, coll. FP.

Description

Apart from colour (see below), we are currently unable to specify any morphological differences between *N. foliosum* and *N. crypticum*. Consequently, the many applicable parts of the description of “*N. foliosum*” by Kato and Pleijel (2002) are not repeated here. Those authors' figures 1A–F,

3A, and 4I refer to *N. foliosum* s. str. (based on depth distribution), whereas their figures 3B, C and 4A–H may refer to either *N. foliosum* or *N. crypticum*.

The holotype of *N. crypticum* n. sp. (spm 12; Fig. 6) is an entire specimen 19 mm in length at 78 segments.

Colour Pale yellowish or dirty white with dark blotches. Eyes dark red to brown. Dorsal cirri without white pigment spots.

Habitat

Currently known only from *Lophelia* reefs at 101–350 m depth.

Distribution

Currently known only from Hjeltefjorden and Trondheimsfjorden in western Norway.

Remarks

Two names previously proposed for species of *Notophyllum* described from Norwegian waters might be synonyms of *N. crypticum*: *N. polynoides* Ørsted, 1845 (species described from Drøbak), and *Trachelophyllum luetkeni* Levinsen, 1883 (Bergen area). For *N. polynoides* no type specimens are known to have been preserved, and no information on depth and habitat are available. For *T. luetkeni* the depth and habitat are also unknown, but in this case there are two syntypes (ZMUC-POL-1014). However, these specimens lack any traces of white pigmentation and it is impossible to know whether or not such marks were present in the live condition. Consequently, we cannot decide whether the syntypes of *T. luetkeni* belong to *N. foliosum* or *N. crypticum* n. sp. From a nomenclatural viewpoint this left us with two alternatives: (1) to select one of the two earlier, available names and fix its taxonomic interpretation with a redescription and, in the case of *N. polynoides*, designation of a neotype; or (2) to treat both names as nomina dubia and propose a new species name in *Notophyllum*. Although our decision can be considered as arbitrary to some extent, we have opted for the second alternative, since there is no positive evidence that either *T. luetkeni* or *N. polynoides* actually denote the same species as our ‘deep form’.

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