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Nothing in (sponge) biology makes sense – except when based on holotypes

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Sponge species are infamously difficult to identify for non-experts due to their high morphological plasticity and the paucity of informative morphological characters. The use of molecular techniques certainly helps with species identification, but unfortunately it requires prior reference sequences. Holotypes constitute the best reference material for species identification, however their usage in molecular systematics and taxonomy is scarce and frequently not even attempted, mostly due to their antiquity and preservation history. Here we provide case studies in which we demonstrate the importance of using holotype material to answer phylogenetic and taxonomic questions. We also demonstrate the possibility of sequencing DNA fragments out of century-old holotypes. Furthermore we propose the deposition of DNA sequences in conjunction with new species descriptions.

Keywords: Porifera, sponges, holotypes, molecular systematics, type material

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INTRODUCTION

Dobzhansky reminds us in his famous 1973 paper (for which our title is a homage) that evolution is the driving force for all biological phenomena (Dobzhansky, 1973). Evolution can be regarded as a continuous process of change. Similarly our understanding of evolution continuously changes as a result of rapid progress in research techniques. Typical examples from sponge evolutionary sciences can be deduced from the numerous changes in phylogenetic hypotheses. Sponge systematics was revolutionized with the introduction of cladistic analyses (e.g. Van Soest, 1987) and the use of molecular methods starting from single gene sequencing (Kelly-Borges *et al.*, 1991) to phylogenomics (e.g. Philippe *et al.*, 2009) (see for reviews and case studies e.g. Boury-Esnault, 2006; Erpenbeck *et al.*, 2012a; Morrow *et al.*, 2013). These molecular tools were soon regarded as the most promising source of phylogenetic characters to further our understanding in sponge evolutionary relationships. Morphological and chemotaxonomic characters displayed shortcomings due to environmental plasticity, homoplasy and lack of complexity, whereas ultrastructure was regarded as too laborious (see e.g. Maldonado *et al.*, 1999; Boury-Esnault, 2006; Erpenbeck *et al.*, 2006; Erpenbeck & Van Soest, 2007; Cárdenas & Rapp, 2013). The subsequent phylogenetic trees of molecular data resulted in few (hexactinellid taxa), to dramatic changes (the other 92% of sponge species) in poriferan systematics (see e.g. Erpenbeck & Wörheide, 2007; Wörheide *et al.*, 2012; Redmond *et al.*, 2013).

Phylogenetic trees provide the basis for exploring and understanding the current patterns and processes observed in all fields of sponge biology, and therefore constitute an important reference for the design of future research (including grant applications). However, publications on character evolution, biochemistry, phylogeny and all other aspects of biology have reduced credibility and impact when the underlying taxonomy is erroneous. In turn, the quality of every phylogenetic tree is dependent on the correct identification of its constituent taxa. While tree reconstruction algorithms advance and facilitate the modelling of molecular evolution scenarios, their underlying data frequently suffer from erroneous taxonomy. For example, DNA sequences submitted to NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank) are not subject to any taxonomic control and frequently bear incorrect taxon names (see e.g. Ashelford *et al.*, 2005; Shen *et al.*, 2013). This is in strong contrast to the function of public DNA repositories, such as the European Nucleotide Archive (http://www.ebi.ac.uk/ena), NCBI GenBank or the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp) as arguably the most important sources for sequences in molecular phylogenetic and taxonomic studies. Different specialized taxonomically curated databases aiming to minimize taxonomic ambiguities

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(e.g. the Sponge Barcoding Database, http://www.spongebarcoding.org; Wörheide *et al.*, 2008) build up a reference backbone, which must rely on taxonomically correct reference material (Wörheide & Erpenbeck, 2007).

The optimal taxonomic reference material for a species is the primary type, or holotype, i.e. the exact specimen used for the species description. The holotype is the single specimen upon which a new nominal species-group taxon is based (International Commission on Zoological Nomenclature (ICZN) 4th Edition, Article 73, 2012), that objectively defines the species concept, and fixes the name proposed by the original author in the original publication. The holotype (or any other secondary type specimen) is usually consulted in morphological taxonomy or systematics, but holotype examination in molecular studies is scarce. In the currently most comprehensive molecular phylogenetic trees for sponges, we find no mention of holotype examination.

Consequently, DNA sequences without unequivocal taxonomic identification are in the majority of cases published in phylogenetic trees and subsequently submitted to public DNA repositories. While the identification of the species from which the DNA was extracted and sequences produced may be subsequently revised and updated, these refinements are not necessarily made globally known, including in GenBank (and other repositories) itself. As such, these taxonomic errors are compounded in subsequent phylogenetic trees that use these sequence databases on the assumption that their original taxonomy was correct.

Conversely, using holotypes for molecular phylogenetic studies confers both taxonomic confidence and more rigour through compliance with the ICZN. Several reasons appear to influence the choice why holotypes are not the primary target for molecular systematic studies. Among the most obvious is the uncertain DNA quality due to age or history of preservation. DNA in the post-mortem cell is subject to a number of types of deterioration such as oxidative and hydrolytic damage, DNA crosslinks and microorganism nucleases (see for an overview Rizzi *et al.*, 2012). These result in DNA fragmentation, amplification inhibition, or base deaminations leading to erroneous genotypes when PCR-amplified under standard protocols (see details in Hofreiter *et al.*, 2001). Destructive processes increase during slow dehydration processes in which the nucleases stay active for some time, or when fixatives were used that trigger DNA-protein crosslinks (e.g. formalin; for extraction protocols see De Bruyn *et al.*, 2011). These destructive processes can be reduced by rapid dehydration (such as preservation in ethanol, silica gel or quick air drying), to inhibit the nuclease activity. However, fragmentation to templates that cannot be amplified with standard primer sets, may prevent the inclusion of holotype sequences in phylogenetic datasets but does not hinder a molecular taxonomic comparison. Here, short DNA markers, 'minimalist DNA barcodes', specifically developed to amplify fragmented DNA templates for molecular taxonomy can facilitate the taxonomic verification of samples by comparison with the holotype prior to publication (Hajibabaei *et al.*, 2006).

Another obstacle for recruiting holotypes in phylogenetic analysis was their accessibility. Discovering where the holotypes are located from historical and foreign language literature, including subsequent taxonomic revisions, potential synonymy, genus transfers etc., are complex and confusing processes. This obstacle is further exacerbated by the antiquity of the current sponge systematics, whereby most genera presently considered valid were fixed by their type species described in the late 19th century, in a scattered literature, and with rarely cited museum specimen numbers, requiring painstaking detective work decades or centuries later (see introductory discussion in (and online updates of) Hooper & Wiedenmayer, 1994). Secondly, accessing the various museums, and then gaining permission to subsample holotypes increases the impediment to source them. However, for sponges there are presently highly comprehensive sources of taxonomic information ranging from the *Systema Porifera* (up to genus level; Hooper & Van Soest, 2002), to dynamic online tools such as the *World Porifera Database* (up to subspecies level; Van Soest *et al.*, 2011) that provide efficient tools to retrieve information on holotypes. Moreover, as DNA can be extracted from minimal amounts of tissue and from every sponge tissue with living cells, DNA sampling in sponges can be considered minimally destructive amongst most Metazoa.

A crude estimation of the number of sequences from holotypes used for sponge systematics undertaken prior to this study yielded less than 80 published sponge holotype sequences (<1% of all described valid sponge species; Van Soest *et al.*, 2012). This is a remarkably low number for a phylum, whose species identification is difficult and challenged by high degrees of environmentally induced plasticity. Consequently, it is obvious that there is a lack of holotype sequences available to undertake precise identifications of taxa for all aspects of sponge research. In this paper we demonstrate the application and advantages of holotype sequences in sponge science based on various case studies, and make a strong argument for their increased use in the future.

MATERIALS AND METHODS

DNA of the following specimens have been extracted in the course of several different projects: MOM INV-22285 (04 0348) (holotype *Heteroxya corticata* Topsent, 1898), BMNH 1881.81.10.21.266 (neotype *Xestospongia testudinaria*), BMNH 1881.81.10.21.267 (associated specimen of Ridley's *Xestospongia testudinaria*), MCZ PORa-6449 and MCZ PORa-6450 (syntypes *Xestospongia muta* (Schmidt, 1870)), ZMB2889 (holotype *Neopetrosia chaliniformis* (Thiele, 1899)), BMNH 1898.12.20.49 (holotype *Neopetrosia exigua* (Kirkpatrick, 1900)), AM Z3867 (holotype *Narrabeena lamellata* (as *Smenospongia lamellata* Bergquist, 1980)), USNM 1231429 (holotype *Stelletta anthastra* Lehnert & Stone, 2014). Specimens were either dry or preserved in ethanol with no further information on other fixatives applied such as formalin. DNA was either extracted using the Qiagen DNeasy Blood & Tissue kit for the recently collected samples (*Narrabeena lamellata, Stelletta anthastra*) or Qiagen QiAmp Mini Kit (*Heteroxya corticata*) following the manufacturer's protocol, or a modified CTAB phenol-chloroform method (Porebski *et al.*, 1997) with the phenol-octanol and RNase solutions steps skipped (*Neopetrosia chaliniformis, Xestospongia testudinaria, Xestospongia muta*). Preferable methods regarding DNA yield and amplification success could not be identified. Fragments of the mitochondrial cytochrome oxidase subunit 1 (CO1, standard barcoding fragment) were amplified using degenerated versions of universal barcoding primers: dgLCO1490 (GGT CAA CAA ATC ATA AAG AYA TYG G)

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and dgHCO2198 (TAA ACT TCAG GGT GAC CAA ARA AYC A) (Meyer *et al.*, 2005) with an annealing temperature of 43°C. Fragments of the mitochondrial cytochrome oxidase subunit 2 (cox2) were amplified using the primers CO2F Por (TTT TTC ACG ATC AGA TTA TGT TTA) and CO2R Por (ATA CTC GCA CTG AGT TTG AAT AGG) (Rua *et al.*, 2011) with an annealing temperature of 40°C. Fragments of ATP6 were amplified using an internal primer set modified from Rua *et al.* (2011) (ATP6_Xt_f1: TAG GGG TAA CTT TGT TAG GG and ATP6_Xt_r1 CCA ATG AAA TAG CAC GAG CC) with an annealing temperature of 44°C. Fragments of the nuclear ribosomal 28S gene (C2-D2) were amplified using the primers 28S-C2-fwd (GAA AAG AAC TTT GRA RAG AGA GT) and 28S-D2-rev (TCC GTG TTT CAA GAC GGG) (Chombard *et al.*, 1998) with an annealing temperature of 50°C. The 25 µL PCR mix consisted of 5 µL 5× green Go*Taq*® PCR Buffer (Promega Corp, Madison, WI), 4 µL 25 mM $MgCl_2$ (Promega Corp, Madison, WI), 2 µL 10 mM dNTPs, 2 µL BSA (100 µg mL^{-1}), 1 µl each primer (5 µM), 7.8 µL water, 0.2 µL Go*Taq*® DNA polymerase (5 u μL^{-1}) (Promega Corp, Madison, WI) and 2 µL DNA template. The PCR regime comprised an initial denaturation phase of 94°C for 3 min followed by 35 cycles of 30 s denaturation at 94°C, 20 s annealing and 60 s elongation at 72°C each and a final elongation at 72°C for 5 min. Alternatively a two-step approach with 4 cycles of 45°C annealing temperature prior to 30 cycles of 50°C were applied. The PCR products were purified with the standard ammonium acetate-ethanol precipitation before cycle sequencing using the BigDye®-Terminator v3.1 (Applied Biosystems) following the manufacturer's protocol. Both strands of the template were sequenced on an ABI 3730 automated sequencer. The poriferan origin of the sequences was checked by a BLAST search against the NCBI GenBank database (http://www.ncbi.nlm.nih.gov). Sequences were base-called, trimmed and assembled in CodonCode Aligner v 3.7.1.1 and subsequently aligned with other representative sequences available from GenBank in MAFFT v7.149b (Katoh & Standley, 2013). All sequences are deposited in the Sponge Barcoding Database (SBD, http://www.spongebarcoding.org; Wörheide *et al.*, 2008) and in NCBI GenBank (see Results and Discussion). Maximum likelihood reconstructions were performed using RAxML 7.2.5 (Stamatakis, 2006) under the GTR model of nucleotide substitution with CAT approximation of rate heterogeneity and 100 fast bootstrap replicates.

RESULTS AND DISCUSSION

Age doesn't (always) matter: BLAST the past

DNA was amplified for all of the above-mentioned type material, including specimens collected in the 19th century. For example CO1 and 28S rDNA sequences were successfully retrieved from *Heteroxya corticata* collected in 1895, which is the type taxon for the family Heteroxyidae Dendy 1905 (SBD# 1152; NCBI accession number KP939318). Likewise, successful amplification of the holotypes of *Neopetrosia chaliniformis* (collected by Sarasin in Sulawesi between 1893–1896 and described as *Petrosia chaliniformis* by Thiele in 1899; SBD# 1153; NCBI: KM030103), and *Neopetrosia exigua* (collected 1898; SBD# 1154, NCBI: KM030104), and type material of the Caribbean barrel sponge *Xestospongia muta* Schmidt (described 1870; SBD# 1155, #1156; NCBI: KM014756), and neotype and associated material of its Indo-Pacific congener *Xestospongia testudinaria*, collected in 1881 (see Ridley, 1884) (e.g. SBD#1157, #1158; NCBI: KM014764; see also publications of Setiawan *et al.* in this volume).

Consequently, there is no reason to assume *a priori* that the antiquity of holotypes, their uncertain preservation history, and the likelihood of strong DNA degradation and fragmentation is a hindrance for successful DNA amplification and sequencing. In fact, DNA quality in old samples might be sufficient for amplification of standard phylogenetic markers if the tissue was stored in ethanol immediately or quickly dried. To our knowledge the first century-old sponge holotype successfully amplified was the holotype of *Topsentia halichondrioides* (as *Trachyopsis halichondrioides* Dendy, 1905), collected 1902, and used for phylogenetic analyses of halichondrid demosponges. Standard phylogenetic markers of 28SrDNA, cytochrome oxidase subunit 1, and the elongation-factor 1-alpha were successfully amplified (e.g. Erpenbeck *et al.*, 2006) (SBD# 1159; NCBI: e.g. AY625676).

Type material, if successfully amplified, is also prone to further contamination, particularly if it is more frequently subject to examination by taxonomists and therefore more likely exposed to contamination, including the metabolomics profile of the taxonomist(s) in question. In particular the use of universal primers such as degenerated CO1 barcoding primers (e.g. Meyer *et al.*, 2005) result in increased yield of non-sponge sequences, which in turn should be easily detectable by phenetic controls like BLAST (Altschul *et al.*, 1990), and these should be followed by (probabilistic) cladistic tree-based methods to ascertain the poriferan origin of the DNA template (see Erpenbeck *et al.*, 2002).

Setting (chemo)taxonomy straight: *Narrabeena* IS a black sheep among Verongida

Species of the order Verongida are frequently subject to biochemical research as they produce bromotyrosines (among other biochemical compounds), which possess bioactive properties of major interest for pharmaceutical research (see for a recent example Mani *et al.*, 2012). Bromotyrosines have been discovered in all genera of Verongida since the morphological revision *sensu* Bergquist & Cook (2002), which suggested an apomorphic nature of this character (Bergquist & Cook, 2002) (see also Van Soest & Braekman, 1999; Erpenbeck & Van Soest, 2007). *Narrabeena* Cook & Bergquist, 2002 is currently classified in the dictyoceratid family Thorectidae, and was erected for *Smenospongia lamellata*, which possesses fibres with a high amount of pith, unlike *S. aurea*, the type species of *Smenospongia*. *Smenospongia* has been regarded as the 'point of closest similarity between Verongida and Dictyoceratida' (Bergquist, 1980). Nevertheless, despite its verongid morphology, *Narrabeena* was placed into Dictyoceratida due to the absence of bromotyrosines. Recent CO1 and 28S rDNA reconstructions, however, resolved *Narrabeena*, investigated in a molecular dataset for the first time, with Verongida (Erpenbeck *et al.*, 2012b). Independent 18S analyses, however, using a different specimen set recovered a *Narrabeena* sample among Dictyoceratida, and implied the need for reanalysis using a conclusive dataset (Redmond *et al.*, 2013). Consequently we analysed the holotype specimen (AM Z3867) from the Australian Museum, Sydney, with molecular methods and yielded a fragment of the C1-D2 region of 28S

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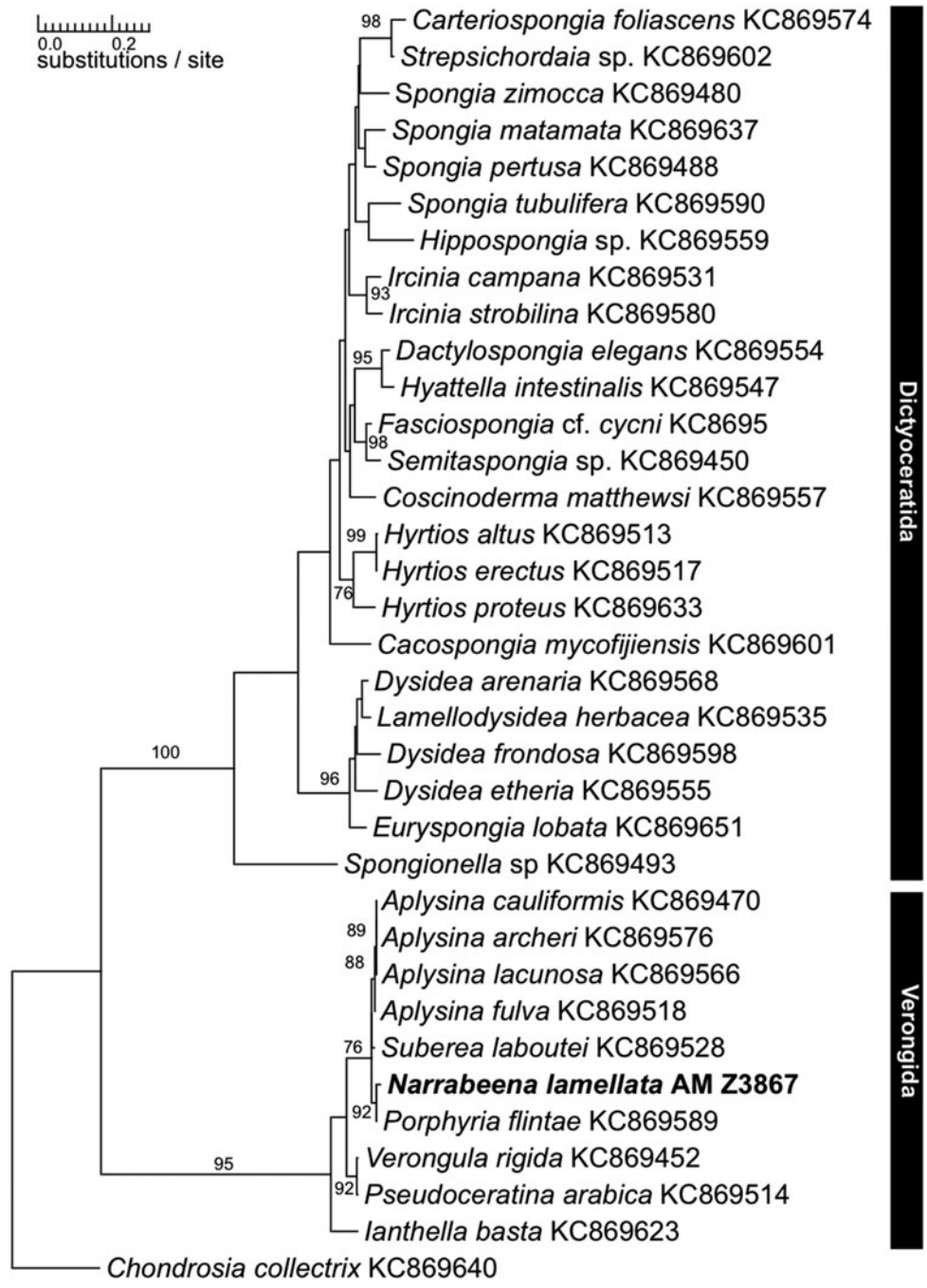

Fig. 1. Maximum likelihood phylogram of the 28S fragment of *Narabeena lamellata* (bold) and representative dictyoceratid and verongid demosponge sequences as retrieved from GenBank. Numbers above the branches are bootstrap support values >70.

rDNA and for CO1 (SBD #1160; NCBI: KP939316). The phylogenetic analyses recovered AM Z3867 within the Verongida in close relationship to *Suberea, Aplysina* and *Porphyria* and distant to the dictyoceratid samples of the dataset with both markers (see 28S rDNA tree in Figure 1). The inclusion of the *N. lamellata* holotype sequence in this analysis therefore clearly shows the verongid relationships of *Narrabeena* and justifies its transfer to Verongida. The analysis also confirms that absence/presence patterns of secondary metabolites in chemotaxonomy have to be evaluated carefully. Besides the independent production of bromotyrosines in other lineages (see review in Erpenbeck & Van Soest, 2007), secondary metabolite production can easily be switched off by mutations in the biosynthetic pathway and its regulatory elements.

Young holotypes of an old phylum: new species with molecular registrations

Molecular methods keep advancing throughout all aspects of sponge biology and molecular taxonomy will likely become the standard for species identification and description in the future. The description of new sponge (and most other metazoan) species will remain predominantly descriptive in the foreseeable future but barcoding approaches and molecularly

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supported museum database platforms (e.g. *Atlas of Living Australia*, see Hooper *et al.*, 2013) also provide various molecular information for the samples. As the costs for DNA barcoding are comparatively low (see e.g. Vargas *et al.*, 2012), sequences can be easily associated with the species descriptions for subsequent analyses, even when the molecular data itself might not be incorporated into species description (which we do not advocate). Examples are the recent publications of Alaskan (Aleutian) sponges (Lehnert & Stone, 2013; Lehnert & Stone, 2014), for which sequences of the mitochondrial CO1 (Barcoding fragment) and 28S rDNA (C1-D2 and D3-D5 fragment; SBD# 1161; NCBI: e.g. KP939317) are submitted to the *Sponge Barcoding Database*. With this information included, phylogenetic trees can be reconstructed by anyone interested, or deducted directly from the *Sponge Genetree Server* (http://www.spongegen-etrees.org; Erpenbeck *et al.*, 2008).

For such a procedure it is evident that samples (or a designated fragment of the sample) are immediately and optimally preserved after collection for molecular purposes in order to keep the DNA amplifiable. Immediate placement in ethanol (as highly concentrated as possible) with ethanol exchange after 24 h (as the seawater dilutes the ethanol) followed by cool storage is among the most practicable and economic methods, with immediate freezing or alternatively the preservation of small sponge crumbles in silica powder (Alvarez *et al.*, 2000), or as well storage in high-salt/DMSO buffer (Seutin *et al.*, 1991; Dawson *et al.*, 1998) also economic and effective.

CONCLUSION

The use of primary type material, and preferably also secondary types for unequivocal verification of the sponge species identification, should be considered for all aspects of evolutionary research, to build a more reliable baseline dataset upon which all new sponge molecular identifications are compared. Although older type material is traditionally infamous amongst many practitioners of molecular barcoding, for the alleged difficulties in achieving conclusive molecular data, we show here that even standard methods may frequently succeed with antiquated specimens. Optimally, molecular identification should be attempted in parallel with comparison with DNA from holotypes. The corollary is that sequence data of non-type specimens without corroboration from type material must be more cautiously interpreted in terms of the power of the evidence they present and the impact on higher systematic interpretation.

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