ZOOLOGICAL SCIENCE **25**: 1253–1260 (2008)

Potential of DNA Sequences to Identify Zoanthids (Cnidaria: Zoantharia)

Frederic Sinniger[1,2]*, James D. Reimer[1,3] and Jan Pawlowski[2]

[1]*Department of Chemistry, Biology and Marine Science, Faculty of Science, University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903-0213, Japan*
[2]*University of Geneva, Department of Zoology and Animal Biology, Molecular Systematic Group, Science III, 30 quai Ernest-Ansermet, 1211 Genève 4, Switzerland*
[3]*Research Program for Marine Biology and Ecology, Extremobiosphere Research Center, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima, Yokosuka, Kanagawa 237-0061, Japan*

The order Zoantharia is known for its chaotic taxonomy and difficult morphological identification. One method that potentially could help for examining such troublesome taxa is DNA barcoding, which identifies species using standard molecular markers. The mitochondrial cytochrome oxidase subunit I (COI) has been utilized to great success in groups such as birds and insects; however, its applicability in many other groups is controversial. Recently, some studies have suggested that barcoding is not applicable to anthozoans. Here, we examine the use of COI and mitochondrial 16S ribosomal DNA for zoanthid identification. Despite the absence of a clear barcoding gap, our results show that for most of 54 zoanthid samples, both markers could separate samples to the species, or species group, level, particularly when easily accessible ecological or distributional data were included. Additionally, we have used the short V5 region of mt 16S rDNA to identify eight old (13 to 50 years old) museum samples. We discuss advantages and disadvantages of COI and mt 16S rDNA as barcodes for Zoantharia, and recommend that either one or both of these markers be considered for zoanthid identification in the future.

Key words: barcoding, molecular taxonomy, Hexacorallia, COI, 16S, mitochondria

INTRODUCTION

Zoanthids are hexacorallian anthozoans belonging to the order Zoantharia. This order is a good example of a taxonomically problematic group (Burnett et al. 1997). All zoanthids, with the exception of the genus *Savalia*, do not secrete a skeleton (Haeussermann, 2003). Numerous morphological identification criteria have been proposed over the last two centuries, ranging from color, sphincter muscle anatomy (Lwowsky, 1913), tentacle number (Herberts, 1972), type and distribution of nematocysts (Ryland and Lancaster, 2004). Thus, depending on the authors, original species descriptions were based on different characters. Moreover, the type specimens are often unavailable for further analyses (poorly conserved, lost or destroyed). This situation has greatly contributed to the abandonment, for the most part, of zoanthid taxonomic studies, even though zoanthids are present in almost all marine environments.

The recently developed concept of DNA barcoding has been highly publicized. This method of organism identification using standardized molecular markers (Tautz et al., 2003, Hebert et al., 2003) offers interesting possibilities for helping to identify organisms by using genetic information in cases where morphology alone is insufficient, due to the existence of cryptic taxa or lack of suitable specimens (Hebert et al., 2003). Fields to which barcoding can be potentially applied are very diverse, ranging from biodiversity assessments (Bucklin et al., 2004) to traceability of commercialized organisms (Ward et al., 2005), through to the linking of different life stages (Hebert et al., 2004a). The key innovative aspect of barcoding is the choice of one or a few standardized markers used for interspecific discrimination. Usually partial cytochrome oxidase subunit I (COI) sequences are used as barcodes. While some groups like birds (Hebert et al., 2004b) and insects (Hajibabaei et al., 2006) are well suited for barcoding utilizing COI, the applicability of this marker to many other animal phyla has not yet been well tested. Additionally, different problems resulting from the use of mitochondrial markers or unclear species concepts (Rubinoff, 2006), intraspecific variation (Meyer and Paulay, 2005) and methodological issues (Brower, 2006) have been shown to potentially confound barcoding efforts. These studies show the dangers of generalisation and the necessity of case studies.

Such problems with barcoding are potentially very problematic for Anthozoa, due to the high conservation in anthozoan mitochondrial genes (Shearer et al. 2002). Hebert et al. (2003) tested only 17 cnidarians for COI barcoding and concluded that this marker was not suitable for Anthozoa.

* Corresponding author. Phone: +81-98-895-8542;
Fax : +81-98-895-8576;
E-mail: fredsinniger@hotmail.com
doi:10.2108/zsj.25.1253

More recently, Shearer and Coffroth (2008) re-examined the situation for scleractinians in detail and showed that sequence conservation makes it impossible to use COI alone to identify most scleractinian species. Huang et al. (2008) also demonstrated that utilizing only COI may not be useful for identifying anthozoans. However, molecular techniques have been shown to be useful in resolving phylogenetic relationships within and between different anthozoan orders (Daly et al. 2003; Sanchez et al., 2003; Fukami et al., 2004; Chen et al., 2002; Berntson et al., 1999). In Zoantharia, mitochondrial and nuclear molecular markers have been used to clarify the phylogeny of the order (Sinniger et al., 2005; Reimer et al., 2006a) as well as to describe new zoanthid species (Reimer et al., 2006b). In an attempt to identify zoanthid species based on standardized molecular markers, in this study we compared partial sequences of two mitochondrial genes, cytochrome c oxidase subunit I (COI) and the large mitochondrial ribosomal subunit (mt 16S rDNA gene) from different zoanthids. We tested both markers, utilizing numerous zoanthid representatives of the most common genera and new zoanthid taxa still in the process of being described. We compare the results obtained to other data such as ecological, geographical, and morphological characters and discuss the applicability of these markers as potential barcodes for zoanthids.

MATERIALS AND METHODS

Sixty-two zoanthid samples of different origins were analyzed in our study. For each zoanthid genus and/or family studied, we used the type species or typical representative species (*Epizoanthus arenaceus* and *E. paguricola* for family Epizoanthidae, *Parazoanthus axinellae* for genus *Parazoanthus* and family Parazoanthidae, *Savalia savaglia* for genus *Savalia*, *Palythoa tuberculosa* for family Sphenopidae, and *Zoanthus* spp. for the family Zoanthidae). Details on locality, depth, and collecting date are available in the supplementary material (Table S1 http://dx.doi.org/10.2108/zsj.25.1253.s1).

To amplify COI, we used the universal primers LCO1490 and HCO2198 (Folmer et al., 1994), and for mt 16S rDNA, we modified the primers 16Sar and 16Sbr (Palumbi et al., 1996). Two additional primers were designed to amplify the short variable region of mt 16S rDNA designated V5 (Sinniger et al., 2005): V5a (5'-ATTCGTAGTGAAGATGCTAC-3') and V5r (5'-TCGCCCCAACCAAACTGTCC-3'). DNA extractions, PCR, and sequencing were performed as described in Sinniger et al. (2005). Additional sequences were obtained from Reimer et al. (2007) (EMBL ALIGN_001072). All sequences are deposited in GenBank under the accession numbers shown in Table S1 (Supplemental Material).

Sequences were manually aligned by using BioEdit (Hall, 1999) and analyzed with maximum likelihood (ML) implemented in the program PhyML (Guindon and Gascuel, 2003). ML analyses were performed with a GTR nucleotide substitution matrix, a gamma 1 invariant model with eight categories, estimated α-parameter, and estimated frequencies of amino acids. Sinniger et al. (2005) clearly demonstrated the basal position of Epizoanthidae within zoanthids. To conserve the limited number of informative sites, we did not include outgroups other than Epizoanthidae. Distance tables for both markers are available as supplementary material (Tables S2 http://dx.doi.org/10.2108/zsj.25.1253.s2 and S3 http://dx.doi.org/10.2108/zsj.25.1253.s3, Supplemental Material)

RESULTS

Cytochrome oxidase I

Fifty-four sequences were used to obtain a nucleotide alignment with 531 sites. Intraspecific variation was observed only in *P. gracilis/tunicans* (detailed below), while all the other species with multiple samples possessed identical sequences (Table S2, Supplemental Material). The COI tree (Fig. 1) distinguished the family Epizoanthidae from the rest of the zoanthids; this result was confirmed by large distance values between species of *Epizoanthus* and other zoanthids (0.116–0.1671; Table S2, Supplemental Material). All the different species can be distinguished with interspecific distances ranging from 0.0019 to 0.0368. *Epizoanthus paguricola* and another undescribed species, *Epizoanthus* sp. "sub-Antarctic", are separated from each other only by a single nucleotide difference (distance: 0.0019); however, clear morphological and ecological differences between these two species leave no doubt as to their specific status.

The second part of the COI tree grouped families Parazoanthidae (genera *Parazoanthus* and *Savalia* (*Gerardia*)), Sphenopidae (genus *Palythoa*), and Zoanthidae (genera *Acrozoanthus* and *Zoanthus*). Due to the short size and high conservation of the sequences used, the topology of this large clade was unresolved. Nevertheless, putative species could be easily distinguished from one another. Among Parazoanthidae sensu stricto, three species (Parazoanthid "Chile", Parazoanthid "CORSARO72", and Parazoanthid "Cape Verde") had sequences considerably divergent from other parazoanthids (distances: 0.021–0.0612). In two cases, molecular distances are supported by clear differences in ecology. Parazoanthid "CORSARO72" grows associated with hexactinellid sponges, and Parazoanthid "Cape Verde" is associated with antipatharians.

Within the same family, nine species currently assigned to genus *Parazoanthus* are all associated with sponges, but their phylogenetic position could not be resolved. Except for *P. parasiticus* from the Caribbean and an undescribed obligately sponge-associated species from Sulawesi (*Parazoanthus* sp. 3 "Sulawesi"), which had identical sequences and could not be distinguished, all the other species could be identified using this marker. Among these species, two very divergent specimens were found, *Parazoanthus* sp. "Senegal" and *P. puertoricense* (distances from other *Parazoanthus*: 0.0171–0.0509; Table S3, Supplemental Material).

Genus *Savalia* was represented by three specimens with identical COI sequences that were clearly distinct from other parazoanthids (0.0133–0.0429); the specific status of these zoanthids will be discussed below (see Discussion).

A clade comprising *P. tunicans* and *P. gracilis* formally belongs to Parazoanthidae, however as suggested in Sinniger et al. (2005) and Reimer et al. (2008), this clade may represent a different family and therefore is referred to here as "Parazoanthidae". The molecular divergences of "Yellow polyps" and an undescribed zoanthid from Madagascar (zoanthid "302") were supported by clear morphological distinctions. Four potentially different species form the sister group to these two zoanthids: two Caribbean samples (*P. tunicans* and *P.* aff. *tunicans*), *P. gracilis*, and an undescribed species from Madagascar (*Parazoanthus* sp. "Mada1"). The two Caribbean samples, previously considered as morphotypes of *P. tunicans*, did not have the same sequence (distance difference=0.0057; see supplementary material), and one sample identified as *P. gracilis* from New Caledonia also had a different sequence than other *P. gracilis* specimens

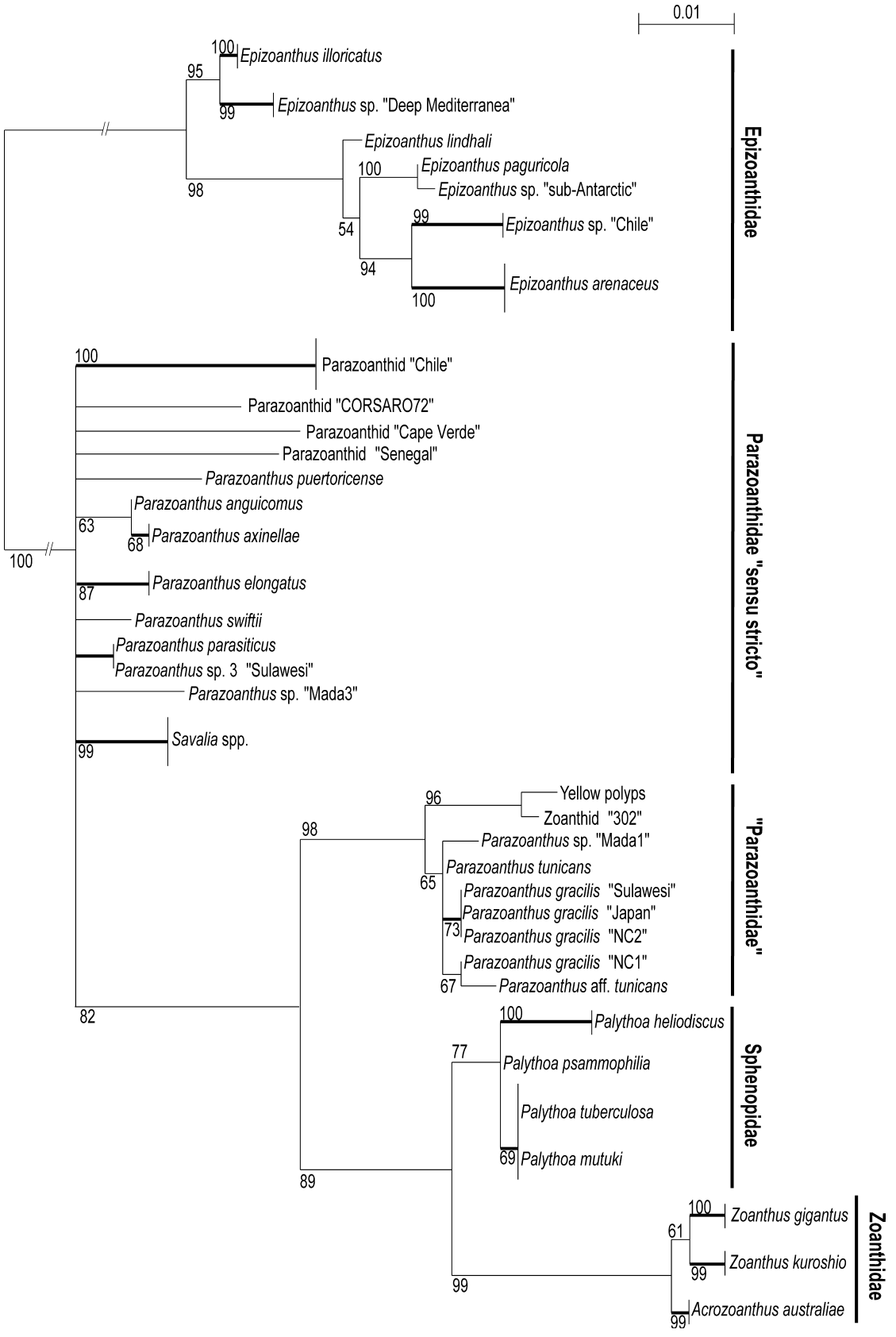

Fig. 1. Maximum-likelihood tree based on an alignment (531 bp long) of mitochondrial COI gene sequences. Numbers above or below branches are bootstrap support values>50%. Thick branches indicate identical sequences.

(distance: 0.0038) (see Supplemental Material).

In the brachycnemic family Sphenopidae, *Palythoa heliodiscus* was clearly divergent from the other *Palythoa* species (distances: 0.0095–0.0114). While all five samples from *P. tuberculosa* and *P. mutuki* had identical sequences, *Palythoa psammophilia* showed a single nucleotide difference from species of the *P. tuberculosa-mutuki* complex. In family Zoanthidae, the situation was also very clear. The two *Zoanthus* species appeared more closely related to each other than to *Acrozoanthus*, and each species was distinct.

Mitochondrial 16S rDNA

An alignment containing 636 sites and 54 sequences was analyzed. The length of the sequences varied from 429–540 bp. The topology of the mt 16S rDNA tree was very similar to the COI tree, although deep nodes were generally less supported, resulting in a less-resolved topology than the COI tree (Fig. 2). However, this did not interfere with the efficiency to distinguish zoanthid species. Like COI, 16S clearly distinguished *Epizoanthus* species from each other, as well as *Epizoanthus* from the rest of the zoanthids, with interspecific distances ranging from 0.0037–0.0487 and distances with other zoanthids ranging from 0.1412–0.2218 (Table S3, Supplemental Material). Moreover, *Epizoanthus* species had longer sequences (524–540 bp) than other zoanthids (429–515 bp), with the exception of the "yellow

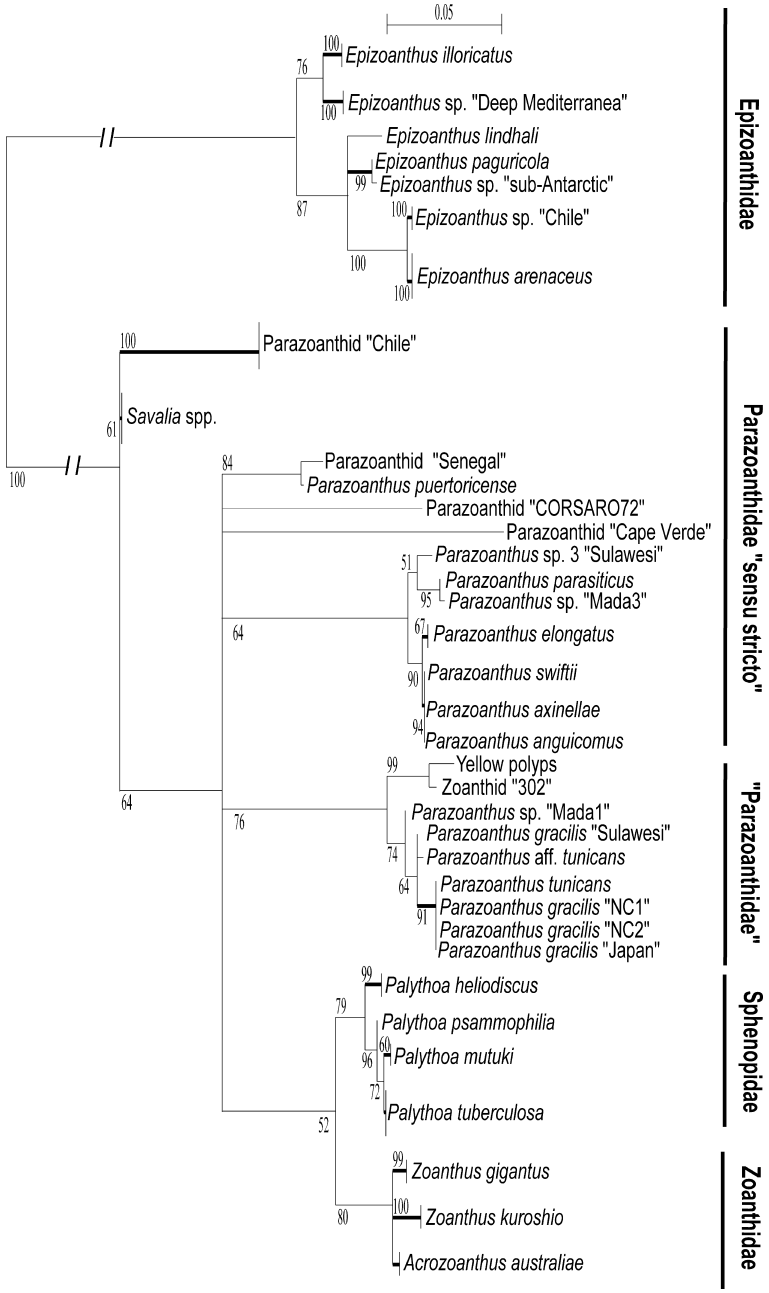

Fig. 2. Maximum-likelihood tree based on an alignment (636 bp long) of mitochondrial 16S rRNA gene sequences. Numbers above or below branches are bootstrap support values>50%. Thick branches indicate identical sequences.

polyps" sequence (528 bp).

The parazoanthid "Chile" and *Savalia* spp. appeared clearly separated from the other Parazoanthidae (distances: 0.0142–0.0802). The two *Savalia* species had identical sequences.

The other species clustered within a large unresolved clade containing six independent branches. Two single-species branches represent respectively the hexactinellid-associated parazoanthid from the Mediterranean Sea (CORSARO 72) and the antipatharian-associated parazoanthid "Cape Verde" (distance from other parazoanthids: 0.0165–0.0802). Parazoanthidae in the wider sense of the term comprises five of the six branches. The sponge-associated zoanthids are respresented as two independent branches. The first branch comprising *P. puertoricense* and *Parazoanthus* sp "Senegal" is clearly separated from other *Parazoanthus* species (distances: 0.02–0.0508). The remaining sponge-associated zoanthids group together; among these, only *P. axinellae* and *P. anguicomus* had identical sequences and could not be distinguished from each other.

The clade "Parazoanthidae" (see COI results section for explanation) is composed of two main clades. The "yellow polyps" and an undescribed zoanthid from Madagascar (Zoanthid "Mada1") are separated from the other Parazoanthidae (including the sister clade) by distances ranging from 0.0170–0.0689. The second clade (distance from other Parazoanthidae: 0.0191–0.0759) includes species associated with hydrozoans and one specimen found on a dead antipatharian skeleton (*Parazoanthus* Mada 1). An intraspecific distance of 0.0063 was observed between *P. gracilis* from Sulawesi and conspecific specimens from New Caledonia (n=2) and Japan. Additionally, *P. tunicans* had a sequence identical to that of most *P. gracilis* specimens. Another sample (*Parazoanthus* aff. *tunicans*) that was morphologically different from *P. tunicans* also has a unique sequence.

Brachycnemic species (Sphenopidae and Zoanthidae) are separated from the other zoanthids by distances of 0.0238–0.1748. Sphenopidae is represented by four species (*Palythoa heliodiscus*, *P. psammophilia*, *P. mutuki* and *P. tuberculosa*). Within *Palythoa*, interspecific variation ranged from 0.0022–0.0131. In family Zoanthidae, three species belonging to two genera were analyzed: *Zoanthus gigantus*, *Z. kuroshio*, and *Acrozoanthus australiae*. No intraspecific variation was observed, and interspecific distances varied from 0.0065–0.0130.

V5 region of the mt 16S rDNA

When analysing the 16S sequence data, the V5 region (150–250 bp) of this gene was seen to have very informative genus-specific indels of relatively short length (Fig. 3). We used this region to examine zoanthids from museum samples that contained old and degraded DNA for which sequences of COI or large fragments of 16S could not successfully be obtained. The two oldest samples from which the V5 region was successfully amplified were collected in 1958. We also amplified sequences from samples collected in 1971, 1972, 1988, 1990, 1991, 1993 and 1995. Our sequences did not present any particular substitutions that would suggest artifactual mutations due to age or conservation methods. The main groups observed with phylogenetic analyses using 16S are visible in the alignment of the V5 region, and two new specific indels in this region were found

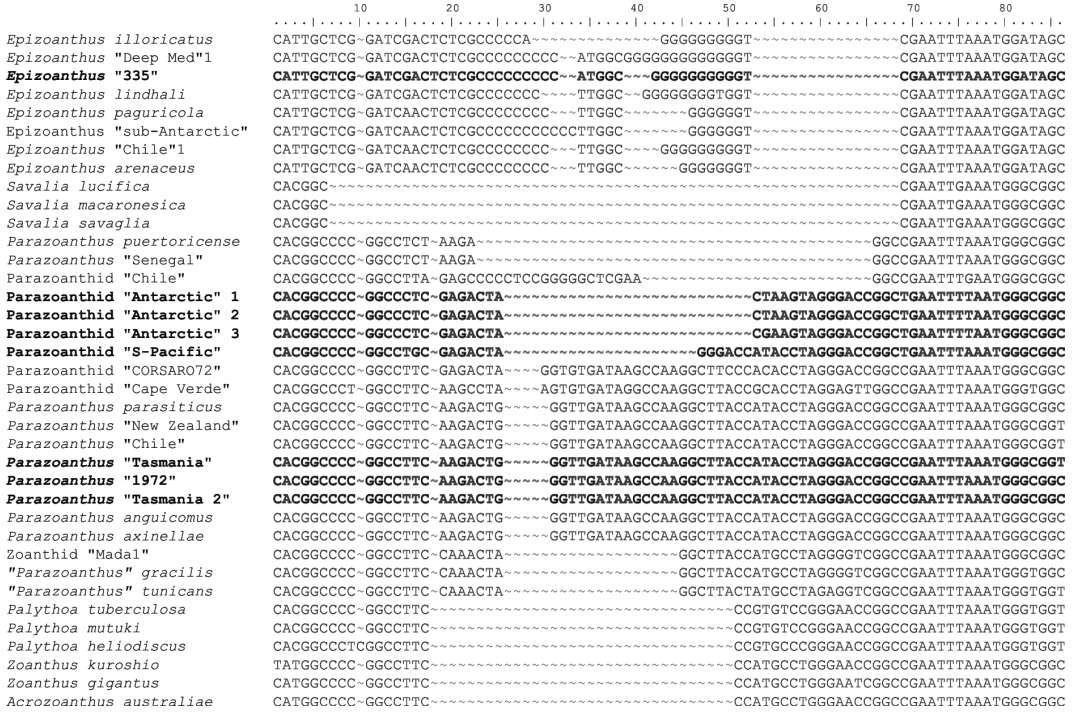

Fig. 3. Alignment of sequences of the V5 region of the mitochondrial 16S rRNA gene from selected zoanthid representatives, including old museum samples (bold).

in old samples of Parazoanthidae.

The first indel groups three epizoic zoanthids growing on gorgonians from the deep Antarctic Sea (Parazoanthidae "Antarctic" 1, 2, and 3). The second indel corresponds to a South Pacific zoanthid epizoic on gorgonians (Parazoanthidae "S-Pacific"). Among other sequences, we were able to place an *Epizoanthus* sp. collected deep in the Indian Ocean (*Epizoanthus* "335") close to the two deep Mediterranean *Epizoanthus*. An Australian zoanthid collected in 1972 (*Parazoanthus* sp. "1972") was identified as a *Parazoanthus* sensu stricto (closely related to the type species of the genus, *P. axinellae*) and had a sequence identical to that of another sample collected more recently (1999) in Tasmania (*Parazoanthus* sp "Tasmania 2"). In the same area of the world, another unidentified sample (*Parazoanthus* sp "Tasmania 1") had the same sequence as *P. elongatus* from Chile and New Zealand, previously analyzed with COI and 16S.

DISCUSSION

Utility of COI and/or mt 16S rDNA

Our results show that, despite the absence of a clear barcoding gap (in a few cases, different species showed identical sequences; in one situation, specimens assigned to the same species showed sequence variation), both the COI and 16S markers are useful for identifying zoanthid at least to the species-group level. While in some cases these markers failed to clearly discriminate between closely related species or species complexes (27/34 species identified with both markers independently and 31/34 with the combination of the two markers), when other simple sampling data such as geographic or environmental information were included, a specific identification was obtained even in problematic cases (33 out of 34 species [=94%] identified with COI and all 34 species [=100%] with 16S). Moreover, described species for which multiple specimens (n>2) were available for COI/16S showed no intraspecific variation (*E. illoricatus*, n=3/3; *E. arenaceus*, n=3/3; *S. savalia*, n=3/3; *Parazoanthus axinellae*, n=4/4; *P. parasiticus*, n=3/3; *Palythoa tuberculosa*, n=17/32; *Z. kuroshio*, n=12/3). The potential intraspecific variability observed in one species is discussed below.

The utilisation of COI has some important technical advantages when compared to 16S. COI is more easily amplifiable with universal primers than 16S (see Materials and Methods). Moreover, the absence of any insertions or deletions in COI facilitates automatic alignment of the sequences obtained. On the other hand, 16S sequences also present distinct advantages. They are slightly more variable in zoanthids than COI and possess insertions and deletions (indels), which are a source of additional taxonomic information. While indels are not always advantageous in aligning sequences, the information contained in these variable regions is very clear and allows easy and rapid distinction between different zoanthid supraspecific clades (Fig. 3). Consequently, if an ambiguous site in a COI sequence (for example due to degraded DNA or antique samples) interferes with species identification, the information contained in insertions is clear even in the case of relatively poor-quality sequences. This may be particularly important when dealing with old museum samples (e.g., holotypes and/or paratypes). As shown by our analyses of the V5 region (Fig. 3), short insertions are more easily obtainable than entire 16S or COI sequences, and thus are more applicable when dealing with old, deteriorated, or formalin-fixed samples.

Problematic situations

There were three cases where only one marker, but not the other, could distinguish between congeners. For example, although *Palythoa tuberculosa* and *Palythoa mutuki* had identical COI sequences, they were distinguishable with 16S. Conversely, no distinction could be made between *Parazoanthus axinellae* and *P. anguicomus* with 16S, while their COI sequences were different. Another example was the sponge-associated *Parazoanthus parasiticus* that could not be distinguished from *Parazoanthus* sp.3 "Sulawesi" in the COI tree; these species were clearly different in the 16S tree. However, in these cases, species could be distinguished by at least one marker, showing that these two markers complement each other very well.

The situation was much more problematic in two other cases. The first case concerns the genus *Savalia*, in which two species were indistinguishable by either the 16S or COI sequences. This situation may possibly be explained by the controversial systematics of this genus. The authors who originally described *Parazoanthus lucificum* (Cutress and Pequegnat, 1960) seemed to not be aware of the existence of *Savalia savaglia*, and examination of type and other *P. lucificum* specimens shows that this species corresponds to the genus *Savalia* (gorgonian associated, skeleton secretion). These conclusions are confirmed by molecular analyses (see Figs. 1 and 2), and therefore here we refer to this species as *Savalia lucifica* (Cutress and Pequegnat, 1960). The main difference between these two species is the bioluminescence of *S. lucifica*. As symbiotic bacteria produce bioluminescence, this potentially environmentally induced character may not be a true "species" marker. *Savalia lucifica* and *S. savaglia* may be conspecific, although their distribution data (*S. lucifica* is found in the northeastern Pacific, *S. savaglia* in the Mediterranean) support the existence of distinct species. The taxonomic position of specimens of *Savalia* aff. *savaglia* from the eastern Atlantic is still under debate (e.g., Ocaña et al., 2006). Although preliminary microsatellite and ITS rDNA data seem to distinguish between these three putative *Savalia* species (F. Sinniger, unpublished data), further studies are necessary to determine whether these differences are due to intra- or interspecific variation.

The second problematic case is the potential intraspecific variability in hydrozoan-associated zoanthids. Only two zoanthids associated with hydrozoans have been formally described, *Parazoanthus tunicans* (Duerden 1900) from the Caribbean and *P. gracilis* (Lwowsky, 1913) from the Indo-Pacific (Herberts [1972] referred *Epizoanthus cnidosus* Tischbierek, 1929 as a synonym of *P. gracilis*). In our study, several different samples (n=6) of hydrozoan-associated zoanthids were analyzed. Two specimens originating from the Caribbean were morphologically and molecularly different from each other; one specimen corresponds to *P. tunicans*, whereas the other was larger and white (designated here *P*. aff. *tunicans*), and was previously identified as a morphotype of *P. tunicans* (Sinniger et al., 2005). These

data suggest that *P.* aff. *tunicans* is an undescribed species. Additionally, *P. tunicans* has COI sequences different from, but 16S sequences identical to, those of most *P. gracilis*.

Four samples of *P. gracilis* were analyzed, two specimens from New Caledonia, one from Sulawesi, and one from Japan. Within *P. gracilis*, one specimen from New Caledonia had a unique 16S sequence, while an Indonesian specimen had a COI sequence different from those of the three other specimens. These divergences could indicate either the existence of cryptic species within this group, or variation in mitochondrial evolutionary rates within this group. More analyses are necessary to clearly understand this potential species complex/clade.

CONCLUSIONS

The levels of divergence observed among different species support the use of COI and 16S sequences in zoanthid taxonomy. This molecular information has already been used in recent taxonomic descriptions and may be useful in the future for species or species-group identification. However, our results also show that molecular data should not be used alone in zoanthid identification, as such data may not distinguish some closely related species. Thus, from a strict barcoding perspective, neither marker is appropriate.

However, if easily accessible ecological, geographical, biological, and morphological data are integrated, we believe that using COI and/or 16S to barcode and help identify zoanthids is well worthwhile. The second objective of DNA barcoding, which is to help indicate potentially new taxa, is relatively well fulfilled with these two markers, the main problem being not evolutionary rate limitations but primarily the taxonomical ambiguities resulting from imprecise zoanthid species descriptions. We believe that the use of these new methods will greatly accelerate our understanding of zoanthid taxonomy and diversity as well as aid in the development of other new and perhaps more accurate methods of species identification in this group.

ACKNOWLEDGMENTS

We thank Prof. L. Zaninetti for her constant support of this research, Dr. H. Zibrowius for his advice as a naturalist, Dr. T. Moldotsova from the Shirshov Institute of Oceanology, Dr. T. Laperousaz from the South Australian Museum and the collectors mentioned in Table S1 for providing us with new samples. We are thankful to the "Fonds Lombard" for financial support of the collecting mission in Madagascar, and SCNAT and the "Basler Stiftung für Biologische Forschung" for the collecting mission in New Caledonia. We also thank the Swiss National Science Foundation for its support and the Rectors' Conference of the Swiss Universities (CRUS) for its financial support; F. Sinniger was partially financed by the Japan Society for Promotion of Science; J. D. Reimer was partially supported by the 21st Century Center of Excellence Program (COE) at the University of the Ryukyus.

REFERENCES

Berntson EA, France SC, Mullineaux LS (1999) Phylogenetic relationships within the class Anthozoa (Phylum Cnidaria) based on nuclear 18S rDNA sequences. Mol Phylogenet Evol 13: 417–433

Bucklin A, Frost BW, Braqdfor-Grieve J, Allen LD, Copley NJ (2003) Molecular systematic and phylogenetic assessment of 34 calanoid copepod species of Calanidae and Clausocalanidae. Mar Biol 142: 333–343

Burnett WJ, Benzie JAH, Beardmore JA, Ryland JS (1997) Zoanthids (Anthozoa, Hexacorallia) from the Great Barrier Reef and Torres Straits, Australia: systematics, evolution and a key to species. Coral Reefs 16: 55–68

Chen CA, Wallace CC, Wolstenholme J (2002) Analysis of the mitochondrial 12S rRNA gene supports a two-clade hypothesis of the evolutionary history of scleractinian corals. Mol Phylogenet Evol 23: 137–149

Collins AG (2002) Phylogeny of Medusozoa and the evolution of cnidarian life cycles. J Evol Biol 15: 418–432

Cutress CE, Pequegnat WE (1960) Three new Zoantharia from California. Pac Sci 14: 89–100

Daly M, Fautin DG, Cappola VA (2003) Systematics of the Hexacorallia (Cnidaria: Anthozoa). Zool J Linn Soc 139:419–437

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3: 294–299

Fukami H, Budd AF, Paulay G, Solé-Cava A, Chen CA, Iwao K, Knowlton N (2004) Conventional taxonomy obscures deep divergence between Pacific and Atlantic corals. Nature 427: 832–835

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696–704

Haeussermann V (2003) Zoanthidea. In "Das Mittelmeer, bestimmungsführer Band II/1" Ed by R Hofrichter , Spektrum, Akademischer Verlag, Heidelberg, pp 501–505

Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PDN (2006) DNA barcodes distinguish species of tropical Lepidoptera. Proc Natl Acad Sci USA 103: 4968–4971

Hall TA (1999) BioEdit: a user-friendly biological sequence alignement editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41: 95–98

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc Lond B 270: 313–322

Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004a) Ten species in one: DNA barcoding reveals cryptic species in the Neotropical skipper butterfly *Astraptes fulgerator*. Proc Natl Acad Sci USA 101: 14812–14817

Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004b) Identification of birds through COI DNA barcodes. PLoS Biol 2: 1–7

Herberts C (1972) Etude systématique de quelques zoanthaires tempérés et tropicaux. Téhtys Suppl 3: 69–156

Huang D, Meier R, Todd PA, Chou LM (2008) Slow mitochondrial COI evolution at the base of the metazoan tree and its implication for barcoding. J Mol Evol 66: 167–174

Lwowsky F (1913) Revision der Gattung Sidisia Gray (Epizoanthus auct.). Ein Beitrag zur Kenntnis der Zoanthiden. Zool Jb Abt Syst 34: 557–614

Meyer CP, Paulay G (2005) DNA Barcoding: error rates based on comprehensive sampling. PLoS Biol 3: e422

Ocaña O, Brito A (2003) A review of Gerardiidae (Anthozoa: Zoantharia) from the Macaronesian Islands and the Mediterranean Sea with the description of a new species. Rev Acad Canaria Ciencias 15: 159–189

Palumbi SR, Martin A, Romano S, McMillan WO, Stice L, Grabowski G (1996) The Simple Fool's Guide to PCR. Kewalo Marine Laboratory and University of Hawaii, Honolulu

Reimer JD, Ono S, Fujiwara Y, Takishita K, Tsukahara J (2004) Reconsidering *Zoanthus* spp. diversity: molecular evidence of conspecificity within four previously presumed species. Zool Sci 21: 517–525

Reimer JD, Ono S, Iwama A, Tsukahara J, Takishita K, Maruyama T (2006a) Morphological and molecular revision of *Zoanthus* (Anthozoa: Hexacorallia) from southwestern Japan with description of two new species. Zool Sci 23: 261–275

Reimer JD, Ono S, Takishita K, Tsukahara J, Maruyama T (2006b) Molecular evidence suggesting species in the zoanthid genera *Palythoa* and *Protopalythoa* (Anthozoa: Hexacorallia) are congeneric. Zool Sci 23: 87–94

Reimer JD, Takishita K, Ono S, Maruyama T (2007) Diversity and evolution in the zoanthid genus *Palythoa* (Cnidaria: Hexacorallia) utilizing nuclear ITS-rDNA. Coral Reefs 26: 399–410

Reimer JD, Sinniger F, Hickman C (2008) Zoanthid diversity (Anthozoa: Hexacorallia) in the Galapagos Islands: a molecular examination. Coral Reefs 27: 641–654

Rubinoff D (2006) Utility of mitochondrial DNA barcodes in species conservation. Conserv Bio 20: 1026–1033

Ryland JS, Lancaster JE (2003) Revision of methods for separating species of *Protopalythoa* (Hexacorallia: Zoanthidea) in the tropical West Pacific. Invertebr Syst 17: 407–428

Ryland JS, Lancaster JE (2004) A review of zoanthid nematocyst types and their population structure. Hydrobiologia 530/531: 179–187

Ryland JS, Brasseur MM, Lancaster JE (2004) Use of cnidae in taxonomy: implications from a study of *Acrozoanthus australiae* (Hexacorallia, Zoanthidea). J Nat Hist 38: 1193–1223

Sanchez AJ, Lasker HR, Taylor DJ (2003) Phylogenetic analyses among octocorals (Cnidaria): mitochondrial and nuclear DNA sequences (lsu-rRNA, 16S and ssu-rRNA, 18S) support two convergent clades of branching gorgonians. Mol Phylogenet Evol 29: 31–42

Shearer TL, van Oppen MJH, Romano SL, Wörheide G (2002) Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). Mol Ecol 11: 2475–2487

Sinniger F, Montoya-Burgoss JI, Chevaldonne P, Pawlowski J (2005) Phylogeny of the order Zoantharia (Anthozoa, Hexacorallia) based on mitochondrial ribosomal genes. Mar Biol 147: 1121–1128

Tautz D, Arctander P, Minelli A, Thomas RH, Vogler AP (2003) A plea for DNA taxonomy. Trends Ecol Evol 18: 70–74

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal- X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24: 4876–4882

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PD (2005) DNA barcoding Australia's fish species. Philos Trans R Soc Lond B 360: 1847–1857

(Received June 2, 2008 / Accepted September 12, 2008)