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Identification and taxonomy of soft-bodied hexacorals exemplified by Chilean sea anemones; including guidelines for sampling, preservation and examination

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The identification of most soft-bodied hexacorals requires morphological and histological examinations of preserved specimens and experience for correct interpretation of the observed features. Poorly preserved or damaged material resulting from improper sampling complicates identification. In many cases the characteristics of the preserved specimens alone do not lead to satisfying results. Living specimens, however, exhibit numerous characteristics which would often allow identification, even in the field. However, most of these characteristics get lost during preservation. Modern techniques and advances in sampling methods allow the acquisition and preservation of a lot of information on the living animal and its habitat.

Using Chilean sea anemone species, it is demonstrated how the work with specimens *in situ* and *in vivo* can help with identification and reveal important morphological–taxonomical, biological, and ecological information. Whenever possible, this information should be part of species descriptions and should be used to create detailed, reliable, tabular identification keys for the laboratory and field. The examples illustrate the urgent need for modern, comparable re-descriptions. In most parts the protocol also applies to other soft-bodied hexacorals.

INTRODUCTION

In the intertidal and shallow subtidal zones of the cold and temperate waters along the Chilean coast, anthozoans constitute a conspicuous and ecologically unique group (Sebens & Paine, 1979). Soft-bodied hexacorals form an important part of the anthozoans. Sea anemones are especially abundant (Guiler, 1959) and presumably are an important factor in local cycles of nutritional matter and in the interaction webs of marine communities. Consequently, they have to be considered in ecological surveys dealing with these benthic communities. However, in the south-east Pacific, soft-bodied hexacorals are one of the most neglected groups in relation to their abundance both in ecological publications and in biodiversity assessments (Moyano, 1995). Ecological studies and *in situ* data, which are necessary for a broader understanding of these animals and of the whole ecosystem, are very scarce.

Reasons for the under-representation of soft-bodied hexacorals in scientific publications may be due to the taxonomic difficulties. Soft-bodied hexacorals lack invariable, solid structures such as a skeleton, spines or spicules. Living specimens with their wealth of characteristics lose important information during the preservation process. Stephenson (1928) doubted whether accurate specific identification from preserved material was always possible. Once an animal is preserved, it is almost impossible to reconstruct its living appearance. The preservable features of these simply organized animals are difficult and time-consuming to access, with histological and morphological work necessary to obtain information for identification. Many characteristics, such as the number of tentacles and mesenteries, the shape of muscles (e.g. sphincter) or the size of cnidae are often highly variable and require experience for correct interpretation (England, 1987). Thus a reliable identification in many cases is only possible for experts (Stephenson, 1928). Poorly preserved or damaged material further complicates the identification process.

Molecular techniques have been used for some taxonomically relatively well-studied groups of sea anemones (Sole-Cava & Thorpe, 1987; McCommas, 1991; McFadden et al., 1997) and will certainly make important contributions to taxonomy in the future. But problems with species identification and systematics still complicate the application of molecular techniques, and the techniques themselves may be problematic for hexacorals (Pinto et al., 2000; Shearer et al., 2002).

In this paper, a detailed modern protocol is presented for recording relevant *in vivo* data and for carrying out optimal sampling and preservation of sea anemones. Most of the suggested techniques are easy to apply and require much less time and experience than traditional histological work. The application of this protocol might help to standardize data recording, improve future species descriptions and re-descriptions, and provide additional information on morphology, biology and ecology. With this information, regional, detailed, tabular identification

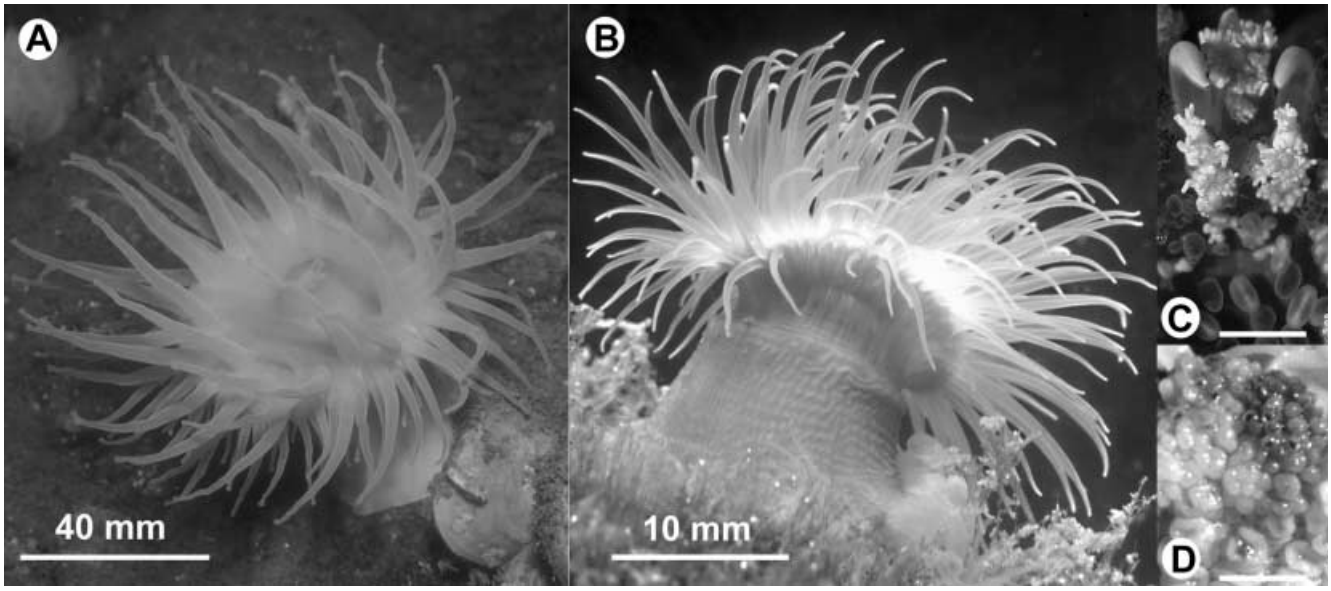

Figure 1. Examples of information that can be obtained from living specimens in the field: (A) Oral view: *in situ* appearance of *Actinostola chilensis*, here between several specimens of the brachiopod *Magellania venosa*; Reñihue fjord, South Chile, 25 m: note typical orientation of tentacles; (B) lateral view: details of column and margin of *Acontiaria* sp.; note division into scapus and distal, delicate capitulum; Quellón, Chiloé Island, South Chile, 10 m; (C&D) details of the upper column and marginal ruff of *Oulactis concinnata*; note transition stages between verrucae and marginal ruff; (C) *in vivo* in an aquarium in Central Chile, scale bar: 5 mm; (D) recently collected, well-preserved specimen, scale bar: 2.5 mm.

keys can be produced for both preserved specimens and animals in the field. These keys should assist benthic ecologists so that these animals receive the attention merited by their ecological importance.

PROTOCOL FOR SAMPLING AND EXAMINATION OF SEA ANEMONES

The following procedures provide a common standard for the collection of shallow-water sea anemones (Actiniaria, Corallimorpharia). Many of the methods also apply to other soft-bodied hexacorals (Ceriantharia, Zoanthidae). For a sound identification of species from a region where hexacorals are poorly known, it is necessary to deliver the collected specimens, ideally with pictures of the same individuals, to an expert. Pictures alone are not enough. For ecological studies, a few identified specimens of every included species should be deposited at a museum to make the identification verifiable for further studies.

(1) In situ *pictures*. Several well focused and correctly exposed pictures of the animals in their habitat should be taken. Oral disc and tentacles (Figure 1A) as well as the column (Figure 1B) should be clearly visible on different pictures of the expanded animal; another picture should show the disturbed/retracted animal.

(2) *Collection* (see also Stephenson, 1928 and Manuel, 1981). Intertidal species can be collected at low tide, subtidal shallow-water species should preferably be collected by SCUBA diving. In some cases it is useful to inject anaesthetic *in situ* so that the animal is relaxed during collection/manipulation. Ideally, specimens should be collected with their substratum using hammer and chisel, provided this does not violate sampling agreements or destroy sensitive habitats. This prevents damage to the pedal disc and reduces the recovery time for animals transferred to an aquarium for further examination. It also reduces atypical behaviour in response to strong disturbance. If specimens have to be separated from the substratum, the base should be carefully and gradually separated from its hold, using a blunt instrument. Buried species are often attached to stones or shells in the substrate; small unattached specimens are best collected using a shovel or trowel and a sieve. These animals have to be collected carefully as they rapidly move downward when disturbed. While collecting the specimens, biotic and abiotic factors as well as any interesting reactions should be noted, such as: reactions to sun or artificial light, vibration, disturbance (e.g. ability and means of contraction); strength of adhesion of the pedal disc to the substratum, adhesiveness of columnar structures, stickiness of tentacles, autotomy of tentacles, etc. The material should already be labelled in the field.

(3) *Observations in the aquarium*. The collected specimens should be transferred into an aquarium with well ventilated and/or regularly changed water. For photography, the ideal aquarium is long, narrow and low, thus allowing easy manipulation and pictures to be taken from different perspectives with a minimum of water between the animals and the camera lens. Well-focused photographs of characteristic details should be taken, including marginal and columnar structures (Figure 1C). One or better two strobe lights facilitate even illumination, keep exposure times short and enable small apertures of the diaphragm. Attention should be paid to colour patterns and their variations

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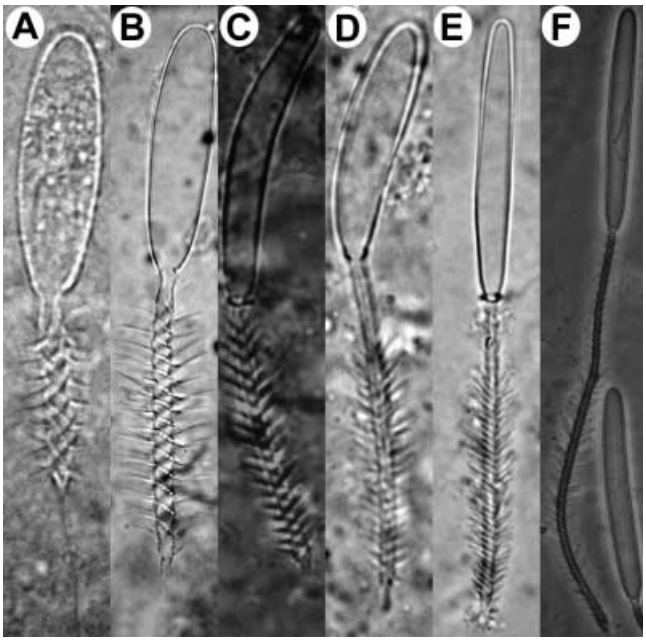

Figure 2. Different mastigophores which, in unfired state, would by most authors be placed in the same category: (A) microbasic amastigophore A; (B) microbasic p-mastigophore D; (C) microbasic p-mastigophore B1a; (D,E) mesobasic amastigophore B2a; (F) macrobasic p-mastigophore B2a (nomenclature *sensu* England (1991)). Note different length and direction of spines, number of windings on shaft, remaining portion of tubule in capsule (F), presence of opening flaps (C–F), presence and length of 'Faltstück' (D–F) etc. Length of capsules A–E, between 20 and 30 μm; F, 50 μm.

on all body parts. Tentacles can often be counted more easily on living than on preserved specimens. Keeping specimens in an aquarium also allows for observations of behaviour, such as time of re-attachment to substratum, mobility, sensitiveness to current or oxygen concentration, inflation of catch tentacles or acrorhagi, or activity rhythms.

(4) *Cnidae data*. The size of at least 40 capsules of each type should be measured per tissue. In each specimen, cnidae should be taken from the same region of the examined tissue. For each type of cnida, the percentage of animals where it was observed should be indicated. These data should be accompanied by mean and standard deviation (Williams, 1996, 1998, 2000). Although pooled values provide a good size range, they cannot readily be treated statistically and are more ambiguous if the species is found to be heterogeneous. Pedal disc diameters of the specimens have to be noted. If fired cnidae cannot be examined, drawn or photographed in the field, permanent slides should be prepared following Yanagi's method (1999): a small amount of ectodermal tissue is scratched from the specimen with the tip of a forceps or a knife and put into a drop of 4% acetic acid or HCl solution on a microscopic slide. After one to two minutes the liquid is drawn off carefully with a tissue. A solution of 1:1 seawater:glycerin that has a few drops each of phenol and formalin per 100 ml is added. The tissue is carefully squashed with a cover slide and sealed several times with nail polish. The coating should be repeated regularly (once after a month and then yearly). Cnidae can then be examined with a light microscope (1000×oil immersion, phase contrast, or interference contrast), drawn and/or photographed, and measured.

(5) *Relaxation*. Before fixation in formalin, the specimens should be relaxed to prevent contraction and secretion of mucus during fixation. Relaxation protocols are numerous (Stephenson, 1928; Pantin, 1946; Manuel, 1981; England, 1987; Moore, 1989; Sebens, 1998), but are not without difficulties (Moore, 1989). An effective protocol for many species is to put them in a dark place into a jar containing a small amount of fresh, cool, well ventilated water, ideally with current. Menthol crystals are scattered on the water surface. Depending on the species, specimens need less than one or up to several hours until they are completely relaxed and cease to respond to tactile stimuli. This relaxation is reversible by replacing the menthol-enriched water with fresh seawater. Other methods of relaxation include the addition of a 3.5–7.5% $MgCl_2$ or $MgSO_4$ solution (see England, 1987; Moore, 1989); but e.g. acontiarian sea anemones tend to macerate after being exposed for more than 30–60 min—as does the tissue after prolonged exposure to menthol.

(6) *Fixation and preservation* (see also Stephenson, 1928). The relaxed animals should be carefully transferred into a solution of 7 to 15% seawater:formalin (concentration depending on the size of the specimens). For a comparison of merits of various fixatives see Dunn (1975). Larger specimens can be prevented from further contraction by injecting formalin into the gastrocoel. This also avoids decomposition of internal structures due to low concentrations of formalin. Alcohol is not recommended as a fixative because the tissue tends either to dissolve or to harden. Transfer to 70% ethanol should not be carried out before the material has been stored two to six months in formalin.

(7) *Tissue for molecular work*. For isoenzyme analysis, frozen tissue is required. If freezing is not possible, tissue for molecular work should be preserved either in a solution of dimethylsulphoxide and sodium chloride (DMSO–NaCl) (Dawson et al., 1998) or in 95% to 100% ethanol. To reduce contamination with symbiont-DNA in zooxanthellate species, tissue from the pedal disc should be used.

(8) *Morphological and histological examinations*. Preserved specimens should be examined in detail (see Stephenson, 1928; Carlgren, 1949; Manuel, 1981). Histological studies are necessary to observe details on muscle and tissue anatomy and distribution of reproductive tissue (see e.g. Pantin, 1946; Humason, 1967; Manuel, 1981).

(9) *Identification*. For basic information on morphology and available identification keys for Actiniaria and Corallimorpharia see Stephenson (1922, 1928) (the latter with detailed information on practical methods, morphology and histology), Carlgren (1949) including a key for genera, den Hartog (1980) and den Hartog et al. (1993). For Zoanthidae see Ryland (1993) including a key for genera; and for

Ceriantharia see Carlgren (1912) and Tiffon (1987). In case of doubt, reference collections or type material have to be examined for comparative purposes. However, one should consider before collecting how many specimens are required and justifiable or if identification can be carried out on the spot.

DISCUSSION AND CONCLUSIONS

Examples of the benefits of in situ *and* in vivo *examinations*

All 22 Chilean species of shallow-water sea anemones I have identified so far can be distinguished at first glance in the habitat or with very few and easy morphological examinations of the living animal. Many of these anemones are distinguished by their characteristic shape, size and colour. The two most common intertidal species, *Phymactis papillosa* (Lesson, 1830) and *Phymanthea pluvia* (Drayton in Dana, 1846) exhibit a similar external morphology, but *in vivo* they are easily distinguishable: *Phymactis papillosa* presents a red, green, blue and brown colour variety and *Phymanthea pluvia* always differs distinctly by its almost invariable bright orange colour and the whitish tips of its vesicles (Häussermann, accepted). Preserved specimens of both species are very similar for the inexperienced observer: the main differences being the histology of the vesicles and one additional type of cnidae in the acrorhagi of *Phymactis papillosa*. These species are a good example of how *in vivo* features can facilitate field identification to non-experts if both species are well studied and variability is known.

Behaviour and reactions to stimuli can also help distinguish Chilean sea anemones. Living specimens of *Bolocerops platei* McMurrich, 1904 and *Bolocera* aff. *occidua* McMurrich, 1893 are similar in appearance, size and colour. Members of both species are unable to retract their tentacles, and have pedal discs that detach readily from the substrata without injury. *Bolocera* aff. *occidua* has longer tentacles in relation to the body than *Bolocerops platei* and can be found in greater depths. Specimens can be distinguished *in situ* mainly because *Bolocera* aff. *occidua* possesses a tentacular sphincter and autotomizes tentacles when collected.

Besides facilitating identification, *in vivo* features also reveal important additional information on biology and morphology. Two Chilean species, *Oulactis concinnata* (Drayton in Dana, 1846) and *Oulactis coliumensis* (Riemann-Zürneck & Gallardo, 1990) bear a marginal ruff of frond-like structures. There has been confusion and speculation about morphology and function of these peculiar structures in the literature (see Häussermann, 2003). Observations on members of these species *in vivo* revealed that the ruff consists of tissue that is much more delicate than that of the scapus and is not adhesive as thought earlier. The clear transition series between adhesive verrucae and the frond-like structures is difficult to see in preserved material (Figure 1C,D).

In the genus *Actinostola* Verrill, 1883 important morphological characters such as cnidae and sphincter size and the presence of stomata and basal tentacular thickenings vary within a species in ways they normally vary between species of different genera (Riemann-Zürneck, 1971). Despite this variation, Fautin (1984) synonymized *Actinostola chilensis* McMurrick, 1904 with the Argentinean and the Antarctic species, basing her decision on cnidae and anatomy of preserved specimens. However, the Chilean species differs significantly in appearance, size, texture and colour (Figure 1A) from several distinct Antarctic species (Rodriguez, in litt., 2003) as well as from the Argentinean species (Roux, in litt., 2003). In addition, members of the Chilean species never brood young in the gastrocoel as described for both the Argentinean and Antarctic species.

Therefore, in the genus *Actinostola*, species distinctions cannot exclusively be based on these traditionally used characteristics of preserved specimens; there is an urgent need for additional distinguishing features (Häussermann, in press).

The colonial nature of *Cereus herpetodes* (McMurrich, 1904) was unreported until recently (Häussermann & Försterra, 2003). This may be because the flat colonies that can be found in the rocky shallow subtidal are strongly attached to the solid rock, and without SCUBA diving it is almost impossible to collect a larger colony without damaging it.

Taxonomy and identification

It is important to differentiate between the systematics of a group of animals, which is the work of experts, and the identification process, which should be possible for all scientists. Data from observations of living animals have to be seen as important additional features, but as far as descriptions are concerned, they can or should not replace morphological and histological examinations. Many features that can be observed on living specimens, e.g. autotomy of tentacles have a morphological base (in this case a tentacular sphincter) and therefore can also be documented on preserved material; but this requires a lot of experience and is often not absolutely necessary for identification. By knowing the fauna of a certain region well, an expert can create a scientifically sound and detailed identification key including characteristic *in vivo* and *in situ* features (see e.g. Sebens, 1998) that are lost during preservation, such as characteristic shape, size, colour, behaviour, habitat, associated species, etc. During identification of sea anemones, one single feature generally is not enough for a decision; more often a combination of characteristics is used collectively. Because of differences between groups, it remains to the expert to evaluate the variability and thus decide about the usefulness of every feature for each genus. A key should offer the possibility to identify both living and preserved specimens, and therefore should combine characteristics of the living animal with morphological descriptions and cnidae data. Due to the importance of combinations of characteristics, a key should not be dichotomous but tabular.

Cnidae data

Cnidae sizes can only be used in some groups as taxonomic features since size ranges of corresponding types of cnida often overlap. Statistics make cnidae data much more useful but cnidae analyses including them are still rare. The few authors who have addressed the problems involved in the use of cnidae sizes as taxonomic features

(Fautin, 1988; Williams, 1996, 1998, 2000; Acuña et al., 2003) conclude that, even when including statistical methods, differences in cnidae sizes should not be used alone as a diagnostic character to distinguish sea anemone species. Acuña et al. (2003) stated that for acontiarian sea anemones other characteristics have to be used to define species precisely, including morphological analysis of the cnidae. The different types of mastigophores are difficult or impossible to distinguish in unfired capsulae and fired cnidae exhibit many structures that might be characteristic for the species; e.g. the relative length of the shaft and of the 'Faltstück', length and direction of spines, and the number of windings on the shaft (Figure 2). Thus they should be pictured in species descriptions and redescriptions when possible.

Application of the protocol

Sampling by SCUBA diving delivers samples in optimal condition and is possible on substrates and at sites, such as steep rocky subtidal, where land-based or vessel-based sampling methods cannot be applied. Due to the superficial low salinity layer in the Chilean fjords, SCUBA diving is the only technique for studying shallow-water species.

The total effect of the appearance of a specimen depends on the simultaneous presence of a number of small details and cannot always be exactly expressed in words (Stephenson, 1928). Photographs of the living organisms record and distribute a lot of easily comprehensible information and help to illustrate difficult verbal descriptions with a minimum of subjective interpretation. Therefore photographs of the specimen and its characteristic details are desirable. Most journals offer free online appendices for colour pictures. Features based on behaviour, associated species, etc. are more difficult to standardize. They are only intended as additional hints rather than used in a decisive way, unless they are very distinct and constant. To avoid different interpretation of reactions, data should be made as comparable as possible: disturbance might be defined by detaching the pedal disc, response to darkness means behaviour in the night. Of course, it is not always possible to apply all suggested steps, e.g. when deep-sea species are sampled and no remotely operated vehicle is available. Nevertheless, one should strive for carefully including as many steps of the protocol as possible. For shallow-water species the necessary equipment is minimal; careful work is crucial and worth the effort.

Tasks for taxonomists

Taxonomists should be able to identify their task organisms not only in preserved state but also alive. One of the tasks of taxonomists working with soft-bodied hexacorals is to redescribe all poorly described species and to make the correlation between the description of the preserved material and the living animals in the field. Modern identification keys will improve the neglect of soft-bodied hexacorals in many ecological studies and diminish erroneous identifications. Cooperative studies of taxonomists and molecular biologists that incorporate both morphological and molecular data will provide much better descriptions and interpretations of the biological diversity and relationship among the groups.

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