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ORAL COMMUNICATIONS

COULD YOU DESCRIBE THREE NEW PHYLA WITHOUT MICROSCOPY?

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Loriciferans, cycliophorans and micrognathozoans are the latest animals groups to be discovered. Without scanning and transmission electron microscopy (SEM, TEM) these microscopic taxa could not have been described as new phyla. They have very different body plans and are not closely related. The Loricifera was described from Roscoff, France, from coarse marine sediments in 1983[1] using light microscopy and TEM. All 36 described species are marine meiofaunal animals. They are the only metazoans found in hypersaline anoxic Mediterranean basins, where they lack mitochondria, and have been found inside cysts in abyssal Pacific sediments. The phylum shares many apomorphic characters with Priapulida and Kinorhyncha and can be included in the Scalidophora. When the Cycliophora was established in 1995 from the mouthparts of marine lobsters, we suggested a relationship to the Entoprocta and Ectoprocta based on the cuticular ultrastructure and life cycle.[2] However, the alternative view that cycliophorans are allied to Rotifera and Acanthocephala is still debated. The life cycle gets more complicated because now cycliophorans are also found on copepods living on the mouthparts of lobsters. Only 2 species are described, but molecular data show that different lobster species host at least 5 species. The Micrognathozoa, described in 2000 from a cold spring in Greenland,[3] occur worldwide, although there is still only one described species. It shows strong affinities with the Rotifera and Gnathostomulida (within the taxon Gnathifera), based on TEM images of the fine structure of the pharyngeal apparatus, where the jaw elements have cuticular rods with osmiophilic cores. The micrognathozoans also have two rows of multiciliated cells forming a locomotory organ, resembling those of interstitial annelids and some gastrotrichs. This character is absent in the monociliated Gnathostomulida and Rotifera. Rotifers always have a syncytial epidermis (Syndermata), a feature the Micrognathozoa lack. Therefore, they are currently placed basally in the Gnathifera, as sister group either to the Gnathostomulida or the Rotifera.

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FROM OPTICAL TO ELECTRONIC MICROSCOPY: TECHNIQUES AND METHODOLOGIES TO STUDY THE DIVERSITY ON FORAMINIFERA

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Marine biodiversity represents about 15% of total global biodiversity.[1] To date, approximately 200,000–500,000 marine species have been described, but these figures are still not definitive.[2] In fact, some groups like Foraminifera have been poorly addressed and their total diversity is not well understood. The Phylum Foraminifera belongs to the Rhizaria Infrakingdom, which represents one protistan

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supergroup.[3] Historically, the study of Foraminifera has been the domain of palaeontologists though, more recently, the study of their recent living counterparts has kindled the interests of foraminiferologists, particularly, for biodiversity, biological, ecological and biomonitoring studies.[4] More than two-thousand recent living benthic foraminiferal species have been reported, and the overall worldwide diversity is estimated to be around three to four thousand.[5] Although microscopy's techniques have been traditionally considered for foraminiferal taxonomical identification, the applications go far beyond it and the recent microscopic development and their implementation have further extended it. The applications of different microscopy's techniques to study the diversity of benthic Foraminifera in a morphological, genetic, cytological and mineralogical perspectives are here presented. Optical to electronic through confocal microscopies allow researchers and scientists to evaluate the overall morphological diversity, to recognize new species, to characterize features of foraminiferal tests (*i.e.*, mineral composition, deformity), to document the cellular ultrastructure (*i.e.*, organelles), to understand physiological processes, and to assign DNA-based Operational Taxonomy Unit to morphospecies.

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MICROSCOPY IN THE STUDY OF MARINE ALGAE BIODIVERSITY

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The word algae include a heterogeneous group of organisms that belong to several supergroups of eukaryotes mainly distributed in aquatic environments. Modern studies focusing on algae need to identify the algal communities at the species level. Here we provide some examples of use light and scanning electron microscopy methods applied to algal research.

In a number of ecological studies, the biodiversity assessment and the identification at the species level is essential. Some species are bioindicator of water quality and changes in specific composition could indicate environmental alterations. Some species could be introduced by human activities deeply modifying native communities. Some microalgae produce toxins and their identification is essential in the management of harmful algal blooms. Some are used in biotechnological applications.

FLUORESCENT AND SCANNING ELECTRONIC MICROSCOPY FOR INVESTIGATING NEMATODES IN EXTREME MARINE ECOSYSTEMS

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Nematodes are among the most abundant and diversified organisms on Earth. Some nematodes are able to cope with extreme environmental conditions, where most of other animals cannot survive.[1] The association between prokaryotes and eukaryotes (through symbiosis) is a winning strategy allowing the survival of extreme fauna[2]. Little is known about nematode/prokaryote interactions in marine environments, and even less in extreme marine ecosystems. In order to unveil nematode-prokaryote interactions in extreme environments it is necessary to combine molecular analyses to microscopic observations.[2] Scanning electron microscopy (SEM) is one of the best procedure to visualise ectosymbionts and their external appearance. However, this technique does not allow the identification of the bacteria visualized. Fluorescence *in situ* hybridization (FISH) is a molecular technique that provides both identification and visualization of bacteria, including endosymbionts. Both SEM and FISH methods required a specific protocol to fix the nematode and bacteria associated immediately after the sampling[2]. The combination of these techniques, together with metabarcoding of 16S rRNA, allows the discovery of unexplored prokaryote–eukaryote interactions in marine extreme environments. Furthermore, SEM and FISH techniques allow the visualization of morphological characters of the host, implementing our knowledge on taxonomy of extreme marine nematodes.

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CONFOCAL AND SCANNING MICROSCOPY FOR THE STUDY OF MEIOBENTHIC BIODIVERSITY IN TROPICAL HABITATS

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Meiofaunal organisms are very abundant and diverse components of benthic ecosystems and their key role in trophic webs is well-recognized.[1] Despite the numerous investigations on meiofauna, a real estimate of their global biodiversity is far to be reached, especially in tropical areas, where the information are often fragmentary.[2] Taxonomical identification of the meiofaunal species is mainly based on minute morphological features that can be detected by means of several types of microscopy techniques and have to be coupled with molecular approach. Among the possible microscopy techniques, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) can provide high-resolution images and highly detailed three-dimensional reconstructions of specimens that may be used to collect important morphological feature for the description of new species, emendation of new taxa and to give a representation of the ecosystem biodiversity. Thus, this study presents an overview of the possible applications of SEM and CLSM in the study of the freshwater and marine meiofauna of tropical habitats and highlights also the potentialities of the study of the nematode morpho-functional traits in ecology.[3-5]

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THE CONTRIBUTION OF MICROSCOPY TO THE STUDY OF MARINE BIODIVERSITY. STRUCTURES AND INTERACTIONS IN PORIFERA AND CNIDARIA

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Microscopy is often necessary for identifying morphological or eco-ethological traits in marine invertebrates. Porifera and Cnidaria are two phyla of relatively simple aquatic metazoans including about ten thousand of species each one. Taxonomy of Porifera is mainly based on the morphology of the skeleton formed by siliceous or calcareous spicules or collagen diversely organized. Moreover, boring sponges can also be identified using the morphology of scars derived from their boring activity on calcareous substrates. The morphology and organization of the skeleton is also an important taxonomic character for several cnidarian taxa. For instance, the shape and size of calcareous sclerites and/or the observation of the internal axis are essential to identify octocoral species. The ultrastructure of the calcareous skeleton is fundamental also for scleractinians and lace corals (Hydrozoa, Stylasteridae). Hydrozoans have highly diverse cnidocysts whose type, size, arrangement and location represent the main taxonomic characters. Histology combined with microscopy are required to study reproductive biology of sponges and cnidarians. In this case microscopy is important to identify patterns in gamete production and to establish eventual correlations with biotic/abiotic factors. Since most of cnidarians feed on small items, microscopy is also crucial to analyse gut contents. Porifera and Cnidaria are also known to be frequently involved in symbiotic relationships with other organisms ranging from algae to protozoans and other metazoans. These associations usually lead to morphological modifications in the partners and the study of these changes sometimes reveals information about the level of integration between them. Microscopy (optical, SEM, TEM) is essential to investigate morphological characters that have taxonomic importance in Porifera and Cnidaria, sometimes leading to the identification of new species or the introduction of new useful characters. Moreover, the study of the microscopic aspects of symbioses can help to clarify the morphological adaptations evolved by the partners.

ANIMAL DIVERSITY AND BIOGEOGRAPHY AT SEM WITH ROTIFERS

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Biogeography and macroecology are ecological disciplines that try to understand the processes underlying statistical patterns of biodiversity, distribution, and abundance of organisms, analysing ecological and historical factors in the relationships between organisms and their environment in a spatially explicit context at different spatial scales.[1] Such studies are quite advanced for larger organisms, with several generalities and commonalities; yet, for microscopic animals we are still far from even having accurate data on species distribution to describe patterns, before producing inference on the processes driving the patterns.[2] For microscopic organisms, the combined use of observations at light and scanning electron microscopy and of DNA sequence data could help filling knowledge on their biodiversity and biogeography, supporting or diminishing the generalities of the patterns and processes known from studies on larger organisms. During the presentation, I will show examples on biodiversity analyses on microscopic animals, mostly rotifers, putting strong methodological emphasis on the procedure to prepare the jaws of these animals for scanning electron microscopy,[3] and strong ecological emphasis on the questions that can be addressed using biodiversity in microscopic animals as examples. I will provide evidence that a quantitative analysis of morphological features from several individuals of multiple populations using geometric morphometrics can provide relevant information on fundamental questions in biodiversity, including species identity and speciation processes, and even biogeographical inference.[4] A useful tool in such use of pictures is the availability of online repositories, as it is commonly done for DNA sequence data in GenBank.[5]

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BIODIVERSITY OF INSECT SPERM

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Recent studies showed that Hexapoda evolved during the Ordovician (ca. 479 Ma).[1] Since then, Hexapoda have become the most species-rich group of organisms on earth with more than one million described species. The insect sperm is commonly made of a head, which is constituted by the acrosome and the nucleus, and a tail, constituted by the axoneme and the mitochondrial derivatives. This pattern can greatly vary among different insect orders and families in terms of presence/absence, shape and dimension of sperm components. In the current contribution we present a slideshow of the different parts of the sperm cells among insects showing their ultrastructural biodiversity. In particular, we compare two sperm models: one holometabolous, represented by the Red Palm Weevil *Rhynchophorus ferrugineus* (Coleoptera: Dryophtoridae) and one heterometabolous, represented by the Pine Bast Scale *Matsucoccus feytaudi* (Hemiptera: Coccoidea). In the first case the sperm is about 100 µm long, with a three-layered acrosome and a nucleus of about 10 µm, two mitochondrial derivatives, two accessory bodies, one well-developed puff-like structure and a typical insect 9+9+2 flagellar axoneme.[2] In the second case the sperm is characterized by the absence of acrosome, centrioles and mitochondria. This latter case is important because in Sternorrhyncha, the suborder to which *M. faytaudi* belongs, a progressive reduction of the sperm motility is observed from Aphidoidea to Aleyrodoidea.[3] *M. feytaudi* shows a recovery of motility thanks to a Microtubular Organizing Centre (MTOC) which produces a flagellum consisting of a bundle of microtubules connected by dynein-like arms.[4]

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THE CONTRIBUTION OF SEM ANALYSIS IN *EX SITU* CONSERVATION PROJECTS CONCERNING PLANT BIODIVERSITY: STUDYING THE ORCHID-FUNGI RELATIONSHIP IN AN *IN VITRO* SYSTEM

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Orchidaceae is a large family of flowering plants that comprises many species, about 26.000, growing in a great diversity of environments and ecosystems. They establish mycorrhizal symbiosis in which diverse soil fungi lie in contact with orchid roots and colonize them. Fungal hyphae form consistent and peculiar structures inside root cells - fungal coils called *pelotons* - and exchange nutrients with their host.[1] Nowadays, orchids are subject to a variety of threats in their natural habitat, but the projects aimed to conserve orchids biodiversity should take account of the highly specific relationship of orchid species with fungi.[2] The objective of this study was to test an *in vitro* mycorrhizal system using plantlets of terrestrial orchids and strains of fungi isolated from orchid roots to improve plant acclimation. Plantlets were obtained from seeds of *Serapias vomeracea* (Burm.f.) Briq. and *Anacamptis laxiflora* (Lam.) R.M. Bateman, Prodgeon & M.W. Chase by asymbiotic germination, *i.e.* without any fungus. Fungi, *Ceratobasidium* sp. and *Tulasnella calospora* were isolated from *S. vomeracea* plants growing in natural habitat.[3,4] Different times of co-cultures were tested and then roots were processed, using Scanning Electron Microscope and Confocal Microscope, in order to evaluate the establishment of a useful mycorrhizal symbiosis. Pelotons have been observed into the cells of root cortex, both in *S. vomeracea* and *A. laxiflora*. Mycorrhizal symbiosis positively affected the growth of plantlets in *ex vitro* conditions, which varied in dependence by orchids species and fungal strains. However, both orchid species showed a significant increase after two months of acclimation.

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POSTERS

MICROPHYTOBENTHIC COLONIZATION OF HARD SUBSTRATA UNDER DIFFERENT HYDRODYNAMIC REGIMES: AN *IN SITU* EXPERIMENT

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In the framework of the Interreg project TRECORALA (TREzze e CORalligeno dell'ALto Adriatico), an *in situ* experiment was set up during autumn 2014 in order to study the potential influence of different hydrodynamic regimes on the microphytobenthic *(i.e.* mainly diatoms with a body size of 20-200 µm) colonization of hard substrata. Twenty artificial collectors (each carrying 10 glass slides) were deployed nearby two of the several rocky outcrops, called 'trezze', which cover the western side of the northern Adriatic Sea. These target geological formations were selected according to the model-derived velocity of bottom water, *i.e.* intermediate and low for S. Pietro (SP) and Bardelli (B), respectively. After 11 (T11) and 36 (T36) days from the deployment, randomly selected slides were retrieved from the collectors at both outcrops and the biofilm on them was studied using confocal and Scanning Electronic Microscopy (SEM). The parallel use of these two techniques allowed to investigate in detail not only the taxonomic composition of the forming biofilm but also its thickness and layer structure. At T11, the biofilm was thicker on SP (>35 µm) than on B slides (<10 µm), suggesting that its formation was faster where the bottom current was more pronounced. While on SP slides a layer of organic matter covered almost uniformly the glass, on B slides it formed patches covered by prokaryotes and a lower number of diatoms. On all slides, the microalgae were mainly represented by tychopelagic species (*Bacillaria paxillifera* and *Ceratoneis closterium*) that, being prone to resuspension, likely reached the artificial substrata more quickly than other diatom taxa. At T36, the biofilm on B slides was thicker and composed of a more diverse and structured microalgal assemblage than at SP *(i.e.* ~30 µm and ~20 µm, respectively), suggesting that the more turbulent conditions at S. Pietro likely exerted a mechanical detachment of these organisms. At both outcrops, tychopelagic diatoms dominated the biofilm on vertical collectors while the taxonomic composition on horizontal slides was more similar to that observed in soft sediments of the Gulf of Trieste.

DETECTION OF A CYTOPLASMATIC POLY(ADP) RIBOSYLASE PROTEIN EXPRESSION BY CYTOLOGICAL ANALYSIS OF *IN VITRO LYMNAEA STAGNALIS* NEURONS *IN VITRO* BY IMMUNOFLUORESCENCE

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Lymnaea stagnalis is a gastropod belonging to the Phylum Mollusca. What makes this gastropod interesting is its central nervous system (CNS), consisting in a central ring of nine ganglia and a pair of buccal ganglia.[1] Several studies have been carried out about different pathways concerning the CNS.[1] Particularly, we focused on a specific enzymes family, PARPs. These enzymes catalyze the poly(ADP) ribosylation reaction, a post-translational reaction.[2] In this work, we focused on PARP4 (or vPARP), a cytoplasmic localization PARP. vPARP has been characterized in several animal models phylogenetically close to *L. stagnalis*. With this knowledge and the ability to consult *L. stagnalis* transcriptome online, the aim of our project is to identify a similar vPARP protein in *L. stagnalis*, defining structural properties and immunolocalization. Through many experiments, involving immunofluorescence reaction, for vPARP immunolocalization and protein interacting with it, we could prove the expression of a similar vPARP protein in *L. stagnalis* nervous system. Given the positive results of our experiments and the presence in literature of works confirming the expression of vPARP in animals phylogenetically close to *L. stagnalis*, next step could be *in vitro* experiments to study possible reactions between vPARP and individual cytoskeletal proteins, to prove that in *L. stagnalis* nervous system vPARP is involved in cytoskeleton organization and remodeling and, consequently, in the regulation of neurogenesis.

L. stagnalis snails have been bred in laboratory, where in an aquarium it has been reproduced their natural habitat and they were fed with lettuce leaves. Before dissection, snail were anesthetized with 10-25% lysterine solution for 10 min.[3] After 10-15 min in an anaesthetic solution, snails are subsequently pinned down in a dissection dish containing ABS saline and their CNS removed under sterile conditions.[3,4] For neurons isolation, the connective sheath surrounding ganglia is eliminated. Later, before individual neurons isolation, nervous system is left for about 20 min in

a mix made up of Trypsin (0.0006 g/mL) and Collagenase (0.0012 g/mL). After enzymatic digestion, Trypsin inhibitor (0.0006 g/mL) is added and allowed to act for about 10 min. Then we passed to the isolation of individual neurons. Once isolated, neurons were placed on L-polylisinate plates, to help nerve cells cohesion. Plates with dissociated neurons were incubated at 22°C in a high humidity environmen.[3] An immunocytofluorescence analysis was performed, by confocal and fluorescence microscopy, to verify vPARP location and other acceptor proteins such as actin and β-tubulin III. For this experiment, isolated *L. stagnalis* neurons cell culture were used. Neurons were fixed with 4% paraformaldehyde in PBS and incubated, after blocking with 5% nonfat dry milk in PBS-T, with anti-vPARP antibodies (polyclonal, developed in goat, Santa Cruz Biotechnology) and anti-β tubulin III antibodies (monoclonal, developed in mice, Santa Cruz Biotechnology). After PBS washing and blocking (2% nonfat dry milk in PBS-T), neurons were incubated for 2 h, at room temperature, with conjugated anti-goat FITC (fluorescein isothiocyanate) antibodies and Pacific Blue conjugated anti-mouse. After PBS-T washing, the neurons were incubated with falloidine rodaminate (to highlight the cell actin in red).

Immunofluorescence experiments on isolated *L. stagnalis* neurons, cultured with growing factors secreted by *L. stagnalis* nervous system to promote axonal growth, have been successful. Protein immunolocalization in growth cones and axonal endings and interaction with cytoplasmic proteins such as actin and β-tubulin III seem to confirm our hypothesis of vPARP key role in neurogenesis. The results show vPARP immunopositivity, in green, in axonal growth cones and in neurites; instead, in red, actin is highlighted in red while β-tubulin III is highlighted in blues.

From immunofluorescence cytological analysis, we observed a strong presence of vPARP at the cytoskeletal level and its important interaction with skeletal proteins such as actin and β-tubulin III. Therefore, once characterized vPARP in *L. stagnalis*, the next step could be to observe vPARP function inside *L. stagnalis* CNS and the physiological events regulated by it. In the literature, there are works focused on the study of vPARP in the CNS of *O. vulgaris*.[5] In this model it has been hypothesized that vPARP regulates the actin polymerization and depolymerization process, an event totally different from the cytosol detoxification process. On the contrary, in *A. californica* a close correlation was found between the long-term memory and vPARP.[6] If we also observe a correlation between vPARP and actin in *L. stagnalis*, we could demonstrate that the protein is involved in the cytoskeleton structural organization and that it acts as a regulator of neurogenesis process; if so, vPARP could also be involved in the regulation of short and long-term memory processes.

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