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Collection, Culturing, Bioimaging and Species Identification of Tardigrade Species in the Vicinity of Albert Hall, UP Diliman

Tardigrades are segmented, microscopic animals that are easily recognizable by their barrel-like body, stubby legs with claws or suckers at the end of each leg, mouse or bear-like face, and slow gait when walking (Italian: *tardi*=slow; *grado*=walker). They belong to the Phylum Tardigrada, which is positioned between Phylum Nematoda and Phylum Arthropoda. They occupy habitats that are periodically or permanently wet, such as marine, freshwater, or limno-terrestrial habitats which include roof gutters and moss cushions.

The most notable feature of tardigrades is their extremotolerance to various adverse conditions, such as dessication, high osmolarity, and changes in pH, salinity, or temperature. Cryptobiosis is an encompassing term for several survival strategies responding to these conditions that cause the shutdown of most or all of the metabolic pathways. The forms of cryptobiosis in tardigrades are anhydrobiosis, cryobiosis, anoxybiosis, and osmobiosis. Respectively, these are survival strategies in response to dessication, freezing, lack of oxygen, and changes in salinity (Bertolani et al., 2004). Anoxybiosis results in bloating and allows the tardigrades to remain viable for only 3-4 days (Kinchin, 1994), but the other strategies result in the formation of a protective tun form that can last up to 4 years (Rebecchi et al., 2006). In their tun form, tardigrades become resistant when exposed to wide variety of extreme conditions, which include vacuums, x-rays, temperatures approaching absolute zero (Ramazzotti and Maucci, 1983), CO_2 and H_2S, temperatures from 120-125^{0}C for a few minutes or 70^{0}C for 1 hour, UV radiation for 2-6 hours, and exposure to ethanol for a maximum of 10 minutes (Rebecchi et al., 2007).

As of the last species count in 2000, approximately 1,100 tardigrade species have been discovered and described worldwide (Zhang, 2006). However, no reports on tardigrade species distribution or even the existence of tardigrades have ever been made in the Philippines. To address this lack of data, this project aims to collect, culture, and identify through different types of microscopy the species of limno-terrestrial tardigrades in the vicinity of Albert Hall, University of the Philippines Diliman.

The first step of the experiment is tardigrade collection. Tardigrades will be collected from moss samples using the method of Nelson (2002). Small amounts of moss from trees will be collected and soaked facedown overnight in Petri dishes filled with water. If the sample contains tardigrades, these will be actively moving in the Petri dish 1-2 days after the initial immersion in water.

The second step of the experiment is culturing. The tardigrades will be cultured using modified versions of the temperate tardigrade culturing techniques of McNuff and Gabriel & Goldstein (2007).

In McNuff's protocol, *Hypsibius dujardini* tardigrades are kept in Erlenmayer flasks with 150 mL Chalkley's Medium with Soil Extract. The tardigrades are fed with 3-5 mL of concentrated unicellular algal cells such as *Chlorella* or *Chlorococcum* cultured in Bold's Basal Medium. Each flask is then sealed with parafilm. They are then subcultured every 4-6 weeks, which is also the approximate time that the algae will be grazed over. The culture flasks are kept between 10-18^{o}C and exposed to photoperiods of L:D 14-10.

In Gabriel & Goldstein's version, the tardigrades are kept in 60 mm. Petri dishes with commercial bottled spring water at room temperature in shaded conditions. The tardigrades are fed *Chlorococcum* at a ratio of 1 vol algal culture to 4 vol tardigrade culture. Every ten days, the tardigrades are allowed to settle and the old medium is decanted out and replaced 4-5 times to clean out the dishes, after which fresh algae is added.

A different culture technique based on that of Pfannkuchen et al. (2007) needs to be used if carnivorous tardigrades such as *Milnesium tardigradum* are discovered. In their study, they cultured the carnivorous tardigrades on Petri dishes kept at 20^{0}C with a thin layer of 3% agar. A 3 mm layer of spring water was added to the agar surface. Rotifers of *Philodina* spp. were cultured separately in spring water and fed *Chlorogonium* green algae. The tardigrades were then fed by adding rotifers twice a week.

These culturing techniques will be modified in the project. The primary technique will be Gabriel & Goldstein's Petri dish culturing, but McNuff's protocol will be followed regarding the tardigrade culture media and photoperiod conditions. The modifications are as follows. Algal EPA will be used instead of Bold's Basal Medium to culture the algae, which will be *Chlorella*. Temperature will be varied for several

batches of tardigrades being reared at the same time to determine the optimal growth temperature for Philippine tardigrades.

Growth conditions will be monitored closely, with pH, water level, and tardigrade activity being observed regularly. If during culturing, periodic anoxybiosis is observed due to lack of oxygen, sealing of the containers with parafilm as advised by McNuff's protocol will not be observed. Instead, the dishes or flasks will be placed in a larger container containing wet cloth or tissue to keep the environment humid and prevent evaporation, while allowing free gas exchange.

As the tardigrades are being cultured, the third step of the experiment, bioimaging, will also take place to document the tardigrades and identify their species. Tardigrades will be visualized initially under dissecting microscopes at 20-40x magnification, then under compound light microscopes that would reach 1000x with the low power objective. Viewing under oil immersion cannot be performed on tardigrades because they are thick animals and will be crushed before the objective can focus properly. Phase contrast microscopy will be used for better viewing of internal organs, but for taking photomicrographs of some structures that are necessary for species identification, fluorescence microscopy can be used. The structures primarily used by tardigradologists for species identification are the bucco-pharyngeal apparatus, claws, cuticle structure, cuticle appendages, and egg ornamentation (Tumanov, 2007). Of these five, two are autofluorescent: the bucco-pharyngeal apparatus and the claws (Pfannkuchen et al., 2007). Wide-field illumination fluorescence technique based on the protocol of Pfannkuchen et al. may be done to make 3D reconstructions of these structures using a fluorescence microscope. This technique provides good resolution and is resistant to diffraction from the tardigrade's thick cuticle.

No fixation or staining is required for the tardigrade samples that are visualized with this technique. To perform wide-field illumination fluorescence, single tardigrade specimens are embedded in polyvinyl-lactophenol on a 1 mm object carrier under a 0.13 mm cover slip. In Pfannkuchen's method, a fluorescence microscope was equipped with an ApoTome and a planapochromat 63x objective. The ApoTome acquires optical sections by performing structured illumination, resulting in image stacks that can be compiled to create 3D images. In UP Diliman, the equivalent of this setup is the DeltaVision Deconvolution Microscope in the National Institute of Physics, which can also produce 3D images of

samples. To ensure that the measurements of structures are valid, at least 15 individuals of each species must be analyzed.

The monograph of Ramazzotti and Maucci (1983) with the most recent compilation of tardigrade species will be the primary reference for taxonomic identification. If the specimens cannot be identified up to the species level, then complete documentation of the species-defining structures will be performed and a new species report based on the format of Tumanov (2007) will be made. Tardigradologists from other universities will be consulted in case the species report is actually of a species that was discovered after Ramazzotti and Maucci's monograph was published.

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