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Digital microscopic imaging of important taxonomic characters of Philippine *Hydraena (Hydraenopsis)* species

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Abstract — **Biodiversity surveys have revealed several new aquatic beetle species of minute size (0.9 mm to 1.8 mm body length). Following modern standards of morphological taxonomy, internal and external microstructures such as aedeagus, ovipositor, spermatheca, gonocoxite as well as the distribution pattern and shape of certain hair structures were used to illustrate the distinguishing characters of new species. As even the smallest diaphragm opening of light microscopes fails to retrieve images of sufficient depth of the field, light microscope images under ZEISS Primo Vert inverted microscope, equipped with ZEIZZ LD Plan-ACHROMAT 20X objective lens, and an OLYMPUS SZ 61 stereomicroscope, were taken at various focus layers using digital adapter LW Scientific MiniVid DCM310 and were then "stacked" using CombineZM software. Different ways of obtaining high quality images were tested and are compared here in.**

Keywords — **Digital microscopic imaging, stacked images, *Hydraena*, Minute Moss Beetle, taxonomic characters**

I. Introduction

Knowledge of the macroinvertebrate fauna of the Philippines is still insufficient especially of those organismic groups that have no direct commercial value. Therefore the research group "Aquatic Biodiversity, Conservation, and Biosystematics of Stream Dwelling Insects & Decapods" of the Ateneo de Manila University aims to contribute to the improvement of the faunistic knowledge of freshwater macroinvertebrates in the global biodiversity hotspot of the Philippines. Therefore, this study aims to provide methodological instruments for precise and time-efficient imaging of important taxonomic characters of such organisms.

A genus of aquatic insects characterized by their very small body size of ca. 1 mm was chosen to test various methods of digital microscopic imaging.

This genus *Hydraena* (Long-palped Water Beetles) belongs to the family Hydraenidae, or Minute Moss Beetles, a cosmopolitan family of 1,380 known species (in 2008) (Jäch and Balke 2008). Although this beetle family is widely distributed (Jäch *et al.* 2005), Hydraenidae, together with Scirtidae, is one of the least explored beetle families on global scale (Jäch and Balke 2008). This could be due to their small size (with the largest of its species only 3.3 mm long), thus requiring special aptitudes to study, compare, and illustrate their distinguishing characters. The focused genus *Hydraena* consists of more than 550 described species, making it the most diverse water beetle genus, but still a high number of undiscovered species is expected (Freitag and Pangantihon 2010; Jäch and Balke 2008).

Previous species descriptions of Asian *Hydraena* in the last decade (e.g., Freitag and Jäch 2007) predominantly used line-drawings, requiring microscopes equipped with an expensive drawing mirror known as "camera lucida". Every drawn line is generated by screening through the three-dimensional object, a procedure costly in terms of time. However, this leads finally to exact and useful illustrations (Fig. 1).

We aim to replace these time-consuming line drawings by using stacked digital microscopic images (Fig. 2) that would allow a time-efficient illustration of all important diagnostic characters of a specimen by use of the available optical instruments.

II. Material and Method

The minute *Hydraena* specimens were initially preserved in 96% ethanol. Under an OLYMPUS SZ 61 stereomicroscope, specimens were dissected in water or lactic acid with the help of insect pins and micro-forceps to separate the taxonomically important parts, namely: aedeagus, ovipositor, spermatheca, gonocoxite, aedeagus, and sternite with spiculum.

The dissected parts were transferred onto a glass slide and embedded in different liquid media (see below).

Figures 1 and 2. Aedeagi of two different *Hydraena scabra* specimens. (1) Conventional time-consuming line drawing, left side lateral view; (2) stacked digital microscopic image, right side lateral view.

Under a ZEIZZ Primo Vert inverted microscope equipped with ZEIZZ LD Plan-ACHROMAT 20X objective lens and a digital camera adapter LW Scientific MiniVid DCM310, a series of photographs were taken at various focus layers from the lowest to the most upper area of the specimen (dissected body part).

The remaining undissected specimens were prepared in a way that all external body appendages were exposed, if ever possible, and glued on entomological paper plates. With the use of the OLYMPUS SZ 61 stereomicroscope equipped with a digital adapter LW Scientific MiniVid DCM310, a series of photographs were taken again at various focus layers from the lowest to the most upper area, but of the entire specimen.

All series of photos of the same object taken at various focus layers were subsequently stacked using the correct weighted function and the stack functions of CombineZM free software (Hadley 2008) to retrieve images with sufficient depth of focus. Images were then enhanced using graphic software, such as Corel Photo Paint Version 10 (Corel Cooperation 2000).

Several experimental approaches were tried referring to (1) the liquid medium (dry (air), water, or lactic acid) wherein dissected parts or entire specimens were embedded, (2) the magnification (HPO, LPO), (3) the mode of stacking offered by CombineZM and (4) the limitation on the number of images of a series processed by the software.

III. Results

The type of media where the dissected parts are embedded, the origin of light source, the number of photographs taken prior to stacking, and the stacking mode used in the CombineZM software, affect the quality of the image obtained. The amount of detail captured in the image is essential for identification and illustration of the species. The resulting images of the respective experimental approaches are summarized in Table 1.

TABLE I
SET EXPERIMENTAL CONDITIONS FOR IMAGING AND SOFTWARE-BASED PROCESSING

	Unstacked single photo	Weighted Average	Stack / Soft stack	
Water	Fig. 4A	Figs. 2, 3A, 4B, 4C	Fig. 4D	Compound light microscope
Lactic Acid	Fig. 5A	Figs. 3B, 5D	Figs. 5B, 5C	
Water	Fig. 6A	-	-	Dissecting microscope
Dry	-	Fig. 6B	Figs. 6C, 6D, 7	

Stacked images of photographs of specimen in water were not obtained as refractive structures of the surface that are taxonomically important were not visible. The single unstacked image should only demonstrate the general limits of the depth of field in stereomicroscopes even when denser media than air are used (Fig. 6A).

Lactic acid dissolves soft tissue or remnants of the gut, ridding the structure of unwanted debris that would obscure important diagnostic features. Figures 2A and 2B are images of aedeagi of *Hydraena* spp. in water and lactic acid, respectively. Out of convenience, lactic acid is used to 'clean' the structure without incurring any damage onto it and to also decrease the amount of time allotted for image enhancement.

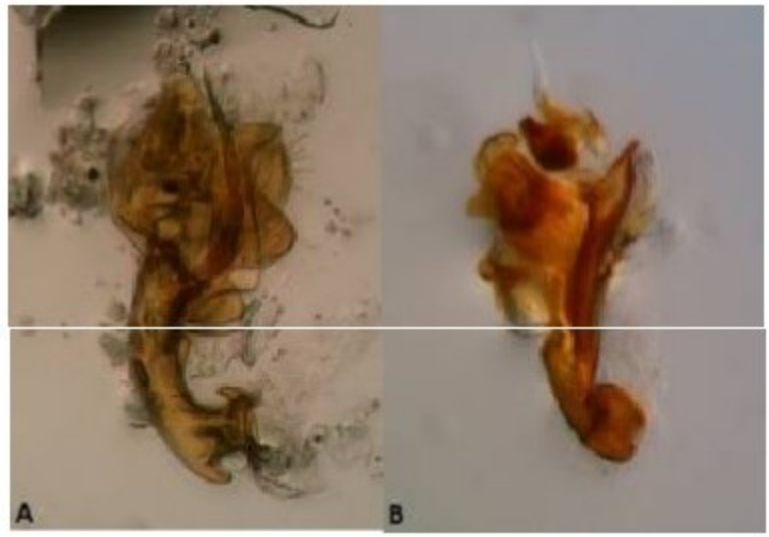

Figure 3. Images of aedeagi in different media after "weighted average" stacking mode of LPO photos. (A) *Hydraena scabra* in water; (B) *Hydraena* sp. in lactic acid.

Figures 4B to 4D exhibit the effects of stacking multiple images under HPO by using either the "soft stack" or "weighted average" mode. Figure 4A is an unstacked photograph of the aedeagus under HPO, and will serve as a control in this set-up.

Figures 4B and C were obtained by stacking layers using "weighted average" mode, differing only in the number of photographs taken. Apparently the number of layers stacked does affect the quality of the image.

CombineZM software is obviously limited in stacking very complex structures by a high number of layers. Fewer layers might still be used to place more focus on a part of the structure. Stacking of ca. 30 layers (which were necessary to capture all structures under HPO) failed. Since parts of the entire structure can only be captured in a limited set of stacked images (Figs. 4C and 4D), a combination of these focused parts may be used to illustrate the specimen. Alternatively, a clear (due to lower number of necessary layers) but a less resolved image can be obtained of the entire specimen under LPO (Figs. 3A, 3B).

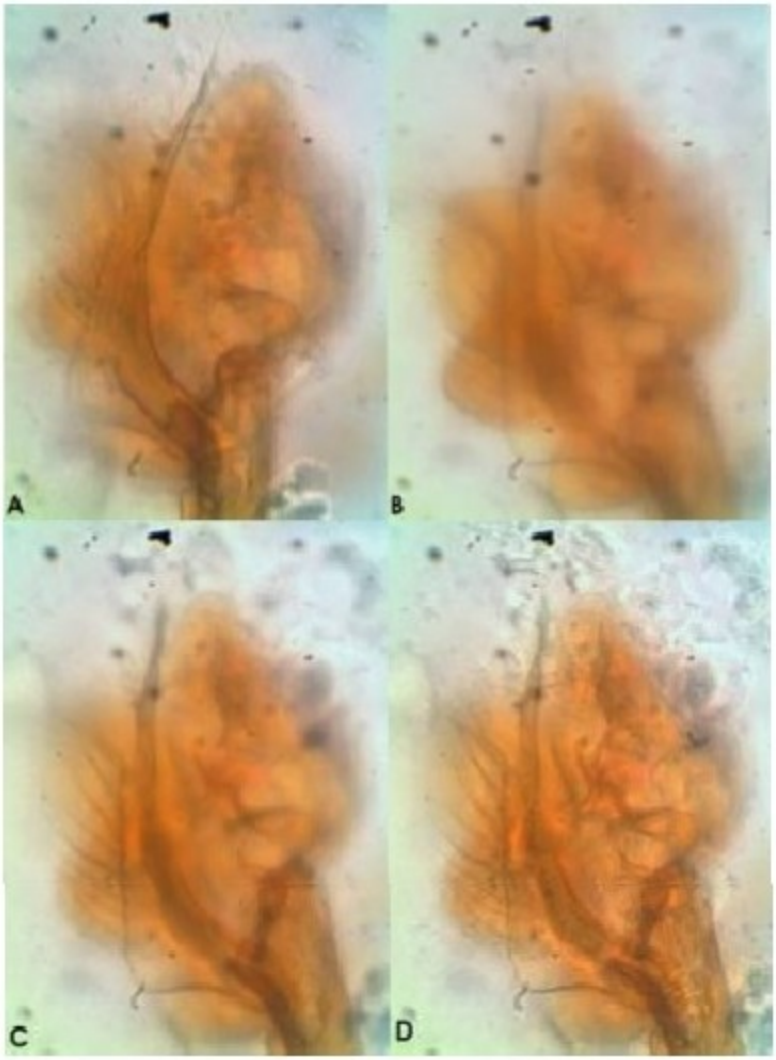

Figure 4. HPO stacked images of the same specimen in water (aedeagus of Hydraena scabra) after different stacking modes. (A) single unstacked image; (B) entire series of 32 photographs stacked by "weighted average" mode; (C) only 10 consecutive photographs of the series stacked by "weighted average" mode; (D) same 10 photographs of the series stacked with "soft stack" mode.

Figures 4C and 4D were obtained from the same number of photograph layers, differing only in the stacking mode used. Figure 4C makes use of "weighted average" mode, while Figure 4D makes used of "soft stack" mode. The latter exhibits more detail compared to the former, but at the same time increases the extent of optical noise.

Twenty digital photographs of the relatively flat (less sterical) gonocoxite of a Hydraena sp. in lactic acid were stacked using a variety of modes: "do stack", "soft stack", and "weighted average" (Figs. 5B to 5D). An unstacked image of the distinguishing character (Fig. 5A) is provided to compare with the stacked images. Resulting image acquired after stacking using "weighted average" mode yielded better results than the other two methods.

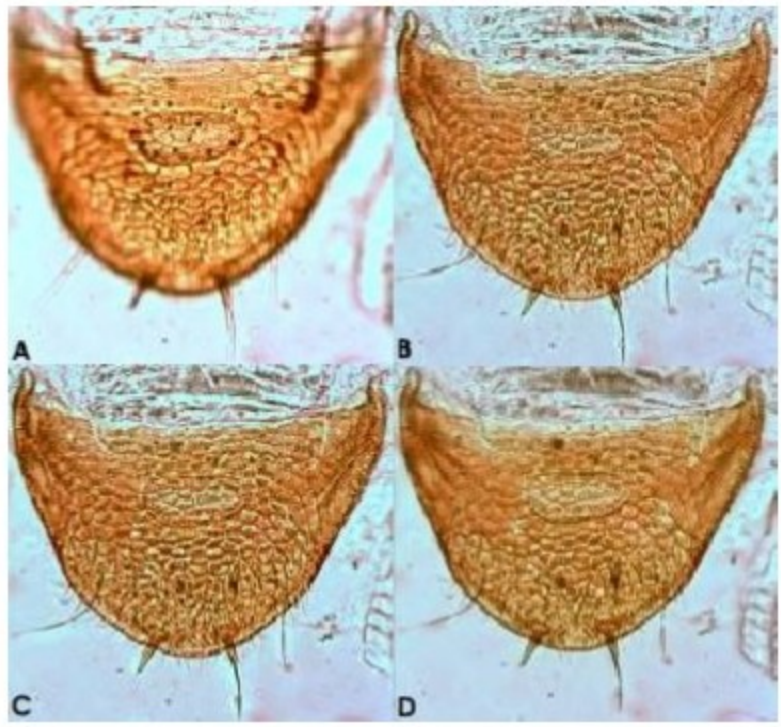

Figure 5. Images of Hydraena sp. gonocoxite in lactic acid. A) single, unstacked image; B) an entire series of 20 photographs stacked by regular stack mode, C) "soft stack" mode and, D) "weighted average" mode.

Figure 6A is an unstacked image of Hydraena sp. in water. Although complete submersion of the specimen reduces reflection of light (Fig. 6A), these reflections might even be necessary to emphasize microstructures on the body surface.

Hydraena sp. in Figures 6B, C, and D were all dry and glued on entomological plates. Each was stacked at different modes: "do stack", "weighted average", and "soft stack", respectively. The "do stack" mode yielded the best image among the three. Using "weighted average" blurred all micro-structures (e.g., elytral punctures), and "soft stack" mode failed to capture the fine distal portions of the specimen (e.g., ends of maxillary palpi and tarsus).

In photographing dried specimens, some reflections (aside from the reflection needed to visualize microstructures) could not be avoided, even by indirect illumination. However, these unwanted reflections can be removed with the use of photo-enhancing software.

Images taken under suitable conditions were enhanced by removal of noise and background (Figs. 2, 6B). Tools of the program Corel Photo Paint Version 10 were used for this purpose.

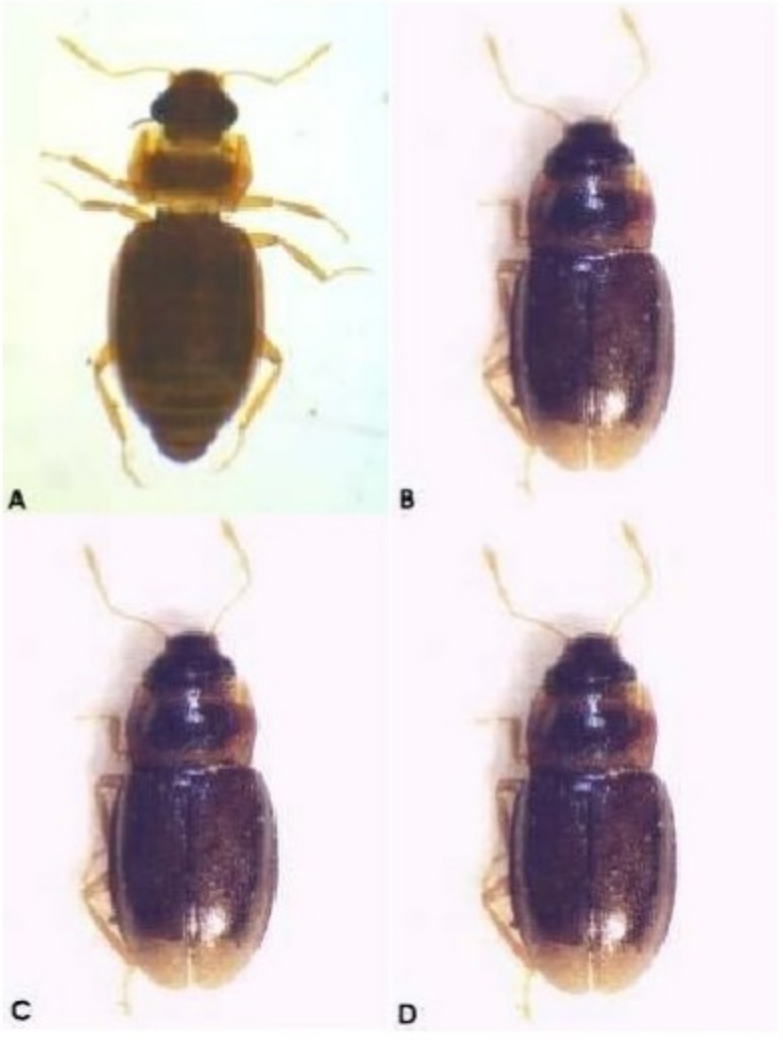

Figure 6. Unstacked and differently stacked images of undescribed *Hydraena* spp. (A) single unstacked stereomicroscopic photo of specimen in water; (B) "weighted average" mode, specimen was dry and glued; (C) "do stack" mode, dry and glued; (D) "soft stack" mode, dry and glued.

VI. Conclusions

Surprisingly, stacked images of photos obtained through the HPO lens did not always produce the best results. This might be due to the inability of CombineZM to stack that many layers. To be able to capture the three-dimensionality of the structure, it is recommended that stacking be done in batches as one region is focused per batch. Combining these focused sections of the entire structure would then yield the desired output.

"Weighted average" mode yielded better pictures for translucent structures with the transillumination light source, while "soft stack" and "regular stack" modes gave better pictures for larger, opaque specimens with the light coming from below. The embedding of dissected parts in lactic acid allows easy removal of disturbing soft tissues from the sclerotized structures without damaging them. This lessens the time allotted for photo-enhancement as these soft tissues have to be removed as they obscure diagnostic features.

Figure 7. A final version of a digital microscopic images of an undescribed *Hydraena* sp. obtained from a dried specimen glued on entomological paper, illuminated by indirect light, after regular stacking and photo-enhancement.

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