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Morphometric Studies in *Enterobryus luteovirgatus* sp. nov. (Ichthyosporea: Eccrinales) Associated with Yellow-banded Millipedes in Puerto Rico

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Abstract. Symbiosis is the association between two non-related organisms. The common yellow-banded millipede, *Anadenobolus monilicornis* (Diplopoda: Spirobolida: Rhinocricidae) and the protist *Enterobryus luteovirgatus* sp. nov. (Ichthyosporea: Eccrinales), a species of hair-like microorganism that inhabits its gut, form a commensalistic relationship. The genus *Enterobryus* was once part of a fungal class (Trichomycetes), but now it is classified as a protist. Other *Enterobryus* species have been reported associated with non-carnivorous arthropod hosts including beetles, crabs and millipedes. Yellow-banded millipedes from Guanica Dry Forest, Puerto Rico were collected to study the prevalence of *Enterobryus* species. A new *Enterobryus* species that inhabits *A. monilicornis* is herein described. Traditionally, *Enterobryus* species are difficult to identify due to high intraspecific variation. Thus, statistical analysis of character measurements is included in an attempt to investigate character stability. Millipedes were dissected; gut linings with attached *Enterobryus* were placed on slides and preserved. Morphometric data of thalli, sporangiospores and holdfasts presented a normal distribution of parameters except for the basal disk width of the holdfast, which showed extreme variation. This character, although used to described *Enterobryus* species is not reliable in *E. luteovirgatus* when using the mean or range values in taxon descriptions.

Key words: Trichomycetes, Diplopoda, *Anadenobolus.*

INTRODUCTION

Enterobryus belongs to the order Eccrinales, once a part of the fungal class Trichomycetes, which are endosymbionts associated with arthropods. These hair-like organisms, as their name suggests, can be found inhabiting the guts of arthropods such as millipedes, crustaceans and insects (Lichtwardt *et al.* 2001). The Eccrinales are present in marine, freshwater and terrestrial habitats and distributed worldwide. The order is a diverse group, which consists of 17 genera and circa 62 described species. Recent molecular data show that these endosymbionts are more closely related to protists than to fungi (Cafaro 2005).

Eccrinales can be found attached to the gut lining of their hosts, held by a secreted disk-like basal holdfast. Morphological characteristics include unbranched, non-septate, multinucleated thalli and sporangiospore production. Typically two types of asexual sporangiospores are produced in most species: (1) thin walled uninucleate, with or without appendages, and (2) thin or thick walled multinucleate, without appendages. Asexual reproduction is the rule in Eccrinales although

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conjugation between separate thalli (interpreted as sexual reproduction) has been observed in *Enteropogon sexuale* (Hibbits 1978).

The first species of Eccrinales, *Enterobrys elegans* Leidy, was described from the millipede *Narceus americanus* in North America (Leidy 1849). Since then, many *Enterobryus* species have been described from millipedes around the world, though many species names have been rejected (Lichtwardt 1986). Currently, *Enterobryus* is the largest genus in the Eccrinales with 25 recognized species inhabiting the gut of crustaceans, insects and millipedes. Only one species, *E. halophilus* Cronin and Johnson, lives in marine environments associated with the gut of mole crabs in the genus *Emerita* (Anomura: Hippidae). Another species, *E. hydrophilorum* (Léger and Duboscq) Manier, 1970, is associated with aquatic beetles; meanwhile the remaining 23 species are all associated with millipedes.

Millipedes are detritivorous invertebrates that prefer to eat decaying plant material rather than living vegetation; they are often found under leaf litter, stones or under the soil surface. Millipedes play important roles in ecosystem nutrient recycling by breaking down organic matter therefore making it easily accessible to other microorganisms for further processing and by stimulating microbial activity (Price 1988). They normally consume approximately 5–10% of annual leaf litter fall (Van der Drift 1975) reaching up to 25% when other microinvertebrates such as earthworms are scarce (Blower 1970, 1974). Microorganisms play a crucial role in the digestive process of millipedes (Anderson and Ineson 1984) although they are poorly characterized and described. Millipedes have symbiotic interactions both externally and internally (Dzingov *et al.* 1982). Externally, they carry bacteria and fungi and can be vectors of microbial dissemination; internally, they harness many symbionts such as bacteria, protozoa, nematodes, trichomycetes and other fungi (Virella Perez and Cafaro 2008). The intestinal bacterioflora is mostly composed of Gram negative aerobic bacteria, mainly bacilli, streptomycetes and coryneform bacteria (Byzov *et al.* 1998). The eukaryotic community in contrast has been poorly characterized. Among nematodes, gregarines, fungi and other protists, the millipedes harbor the specialized symbiont *Enterobryus* (Ichthyosporea). The purpose of this study is to further broaden the diversity of *Enterobryus* by the morphological description of a new species inhabiting the yellow-banded millipede *Anadenobolus monilicornis* (Porta, 1876). Also, utilizing measurements of key structures, we introduce the use of statistical data analysis to assess the distribution of these values, which provide a more accurate description of the species and help identify the more stable characters.

MATERIALS AND METHODS

Collection

Anadenobolus monilicornis specimens were collected by hand turning rocks and removing leaf litter on Jaboncillo Beach Guanica Dry Forest, Puerto Rico (17°57′13.06″N, 66°54′16.54″W) and placed in plastic containers. Each container was filled with leaf litter and sprayed with distilled water occasionally to maintain humidity. In order to reduce population stress the number of individuals was limited to 20–23 individuals per container. In the laboratory a total of 60 individuals were measured and sexed.

Dissection

Individuals were placed on a clean glass petri dish and dissected under a stereomicroscope (Olympus SZ61) with a scalpel by pressing the head towards the dish and cutting it off; then counting 3–4 segments from the end, the rear was also cut. Utilizing forceps, the whole intestinal tract was pulled slowly and gently from within the exoskeleton; placed in a different clean glass petri dish and quickly hydrated with distilled water.

Utilizing ethanol-sterilized scalpels and forceps, cuts were made on the surface of the hindgut exposing the interior side, where the presence of *Enterobryus luteovirgatus* was recorded. The hindgut was cut into smaller pieces and transferred to a drop of water on microscope slides for direct observation under a light compound microscope with phase contrast (Olympus CX41). Slides were preserved by infiltrating lactophenol cottonblue (0.05% w/v) under the cover slip; cleaned and sealed with clear finger nail polish.

Scanning electron microscopy (SEM)

The hindgut was dissected as mentioned above and fixed in 1.5 mL of 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The sample was dehydrated in an increasing series of ethanol dilutions (10–100% solutions) prior to critical point drying. Finally, hindgut samples were coated with gold-palladium and examined under SEM (JEOL JSM-5410LV) at the Microscopy Center of the University of Puerto Rico at Mayagüez campus.

Statistical Analysis

Photomicrographs and measurements of key structures of *Enterobryus luteovirgatus* were taken at the Microscopy Center of the University of Puerto Rico at Mayaguez with Spot Advanced Insight Camera (3.2.4 for Windows). Data were organized into seven categories according to defining characters in the genus *Enterobryus*. Holdfast: (i) stalk length, (ii) stalk width, (iii) basal disk width; Sporangiospores: (iv) length, (v) width; Thallus: (vi) length, (vii) width (Table 1). A frequency histogram was generated for values in each category. Each histogram was assessed both, visually and statistically and compared to a normal distribution curve. Unimodality and lack of skew was determined for each category. Descriptive statistics for each category were generated and each set was analyzed in Minitab ver. 16.1.1.

RESULTS AND TAXONOMY

Enterobryus luteovirgatus sp. nov. Cafaro and Contreras (Fig. 1)

Immature thallus short, straight at the base. Mature thallus elongated, up to 2.1 mm long, 6–23 µm wide; cytoplasm granulose. Holdfast attached at the base of the thallus, 2–15 µm long, stalk 3–12 µm wide, basal disk irregular, flat to slightly convex, 10–35 µm wide. Sporangiospores thin-walled and multinucleate (type A), 20–116 µm × 6–31 µm. In hindgut of Spirobolida (Diplopoda), also attached to the cuticle of nematodes that inhabit the hindgut of the millipede.

Etymology. Host characteristic, referring to the yellow bands on *Anadenobolus monilicornis*.

Specimens examined. Puerto Rico. Guanica: Playa Jaboncillo, under litter from trees near a mixed forest, prepared from *Anadenobolus monilicornis* (Porta, 1876) (Diplopoda: Spirobolida), microscope slides KCV-Org-1 through KCV-Org-60, 21-23-XII-2011. Materials deposited at the Harold W. Manter Laboratory of Parasitology, University of Nebraska-Lincoln, Lincoln, NE, USA included host vouchers, slides and, ethanol-preserved specimens for future DNA work under the accession number P-2013-010. Holotype (KCV-Org-18a) under collection number HWML49781 and paratypes under HWML49782 and HWML49783, respectively.

Remarks. The holdfast (Fig. 1A–E, L) varies in shape and size, flat to slightly convex, with a short stalk and a somewhat circular basal disk. The basal disk sometimes is proportionate to the width of the stalk, while in other cases it extends across the hindgut lining following its curvature. Though the basal disk seems to have a circular area over the attached surface, this area is not often uniform; thus, sometimes forming an irregular polygon in relation to the surface area (Fig. 1B–C). The width of the stalk most of the time is thinner than the thallus and the radius of the basal disk. Thalli are usually hyaline, having a granular material and thin walls. The thalli have more or less consistent width in the range of 6.00–23.00 µm; although the length is very variable (up to 2.1 mm) depending on thallus development. The width stays constant throughout the majority of the thallus length with gradual widening at the terminal end or basally when approaching the holdfast structure attached to the gut lining. Only type A sporangiopores (Lichtwardt 1954, 1958) (Fig. 1H–K) were observed in *E. luteovirgatus*, which are characteristic of most Eccrinales and are believed to be secondary infestation spores. They are thin-walled, multinucleated sporangiospores, which contained more than 4 nuclei (up to 18) depending on the length of the spore; the cytoplasm has a condensed, granular material. Sporangiopores are noted to grow in linear chains, at the distal end of the thallus ranging from 6–31 µm in width to 20–116 µm in length.

An analysis of the frequency distribution of measurements of key structures in *E. luteovirgatus* (Table 1) shows that most of them (except for the holdfast basal disk width) follow an unimodal distribution (Fig. 2). Only data for three of the seven categories were nor-

Table 1. Statistical data: key-structures measurements of *Enterobryus luteovirgatus* associated with *Anadelobolus molinicornis*.

Structure	N	Mean (µm)	Median (µm)	Maximum (µm)	Minimum (µm)	SD	p-value
(i) Holdfast stalk L	28	8.26	7.50	15.00	2.00	3.21	0.261
(ii) Holdfast stalk W	29	6.62	6.00	12.00	3.00	1.89	0.173
(iii) Holdfast basal disk W	35	22.11	22.00	35.00	10.00	6.91	0.525
(iv) Sporangiospore L	67	56.27	53.00	116.00	20.00	19.66	0.020
(v) Sporangiospore W	70	15.01	15.00	31.00	6.00	3.77	< 0.005
(vi) Thallus L	15	1650.00	192.5	2922.00	175.00	758.6	0.167
(vii) Thallus W	172	13.07	13.00	23.00	6.00	3.04	< 0.005

L = length, W = width
Holdfast: (i) stalk length, (ii) stalk width, (iii) basal disk width; Sporangiospores: (iv) length, (v) width; Thallus: (vi) length, (vii) width.

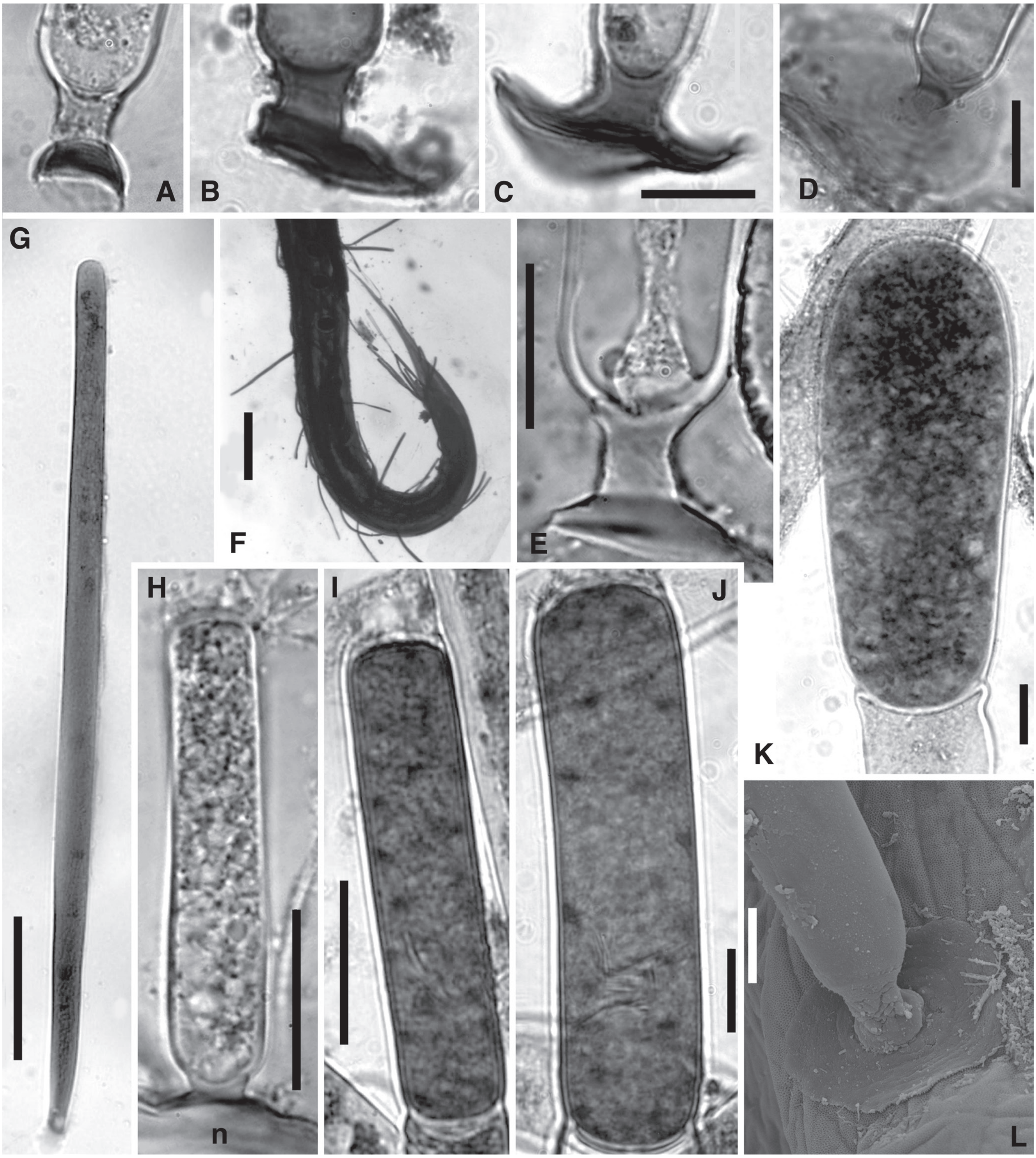

Fig. 1A–L. *Enterobryus luteovirgatus.* **A–E** – holdfast variation: basal disk with different shapes depending on secretion pattern; **F** – multiple thalli attached to a nematode in the gut; **G** – young developing thallus detached from a nematode; **H** – a newly attached multinucleated sporangiospore with a disk-like holdfast cemented on a nematode cuticle (n); **I–K** – multinucleated sporangiospores at distal end of thalli; **L** – SEM microphotograph of thallus attached to the gut lining with a secreted circular basal disk. All microphotographs were taken after lactophenol-cotton blue fixation. Scale bars: C = 10 µm (valid for A–C), D = 10 µm, E = 15 µm, F–G = 100 µm, H = 15 µm, I = 25 µm, J–L= 10 µm.

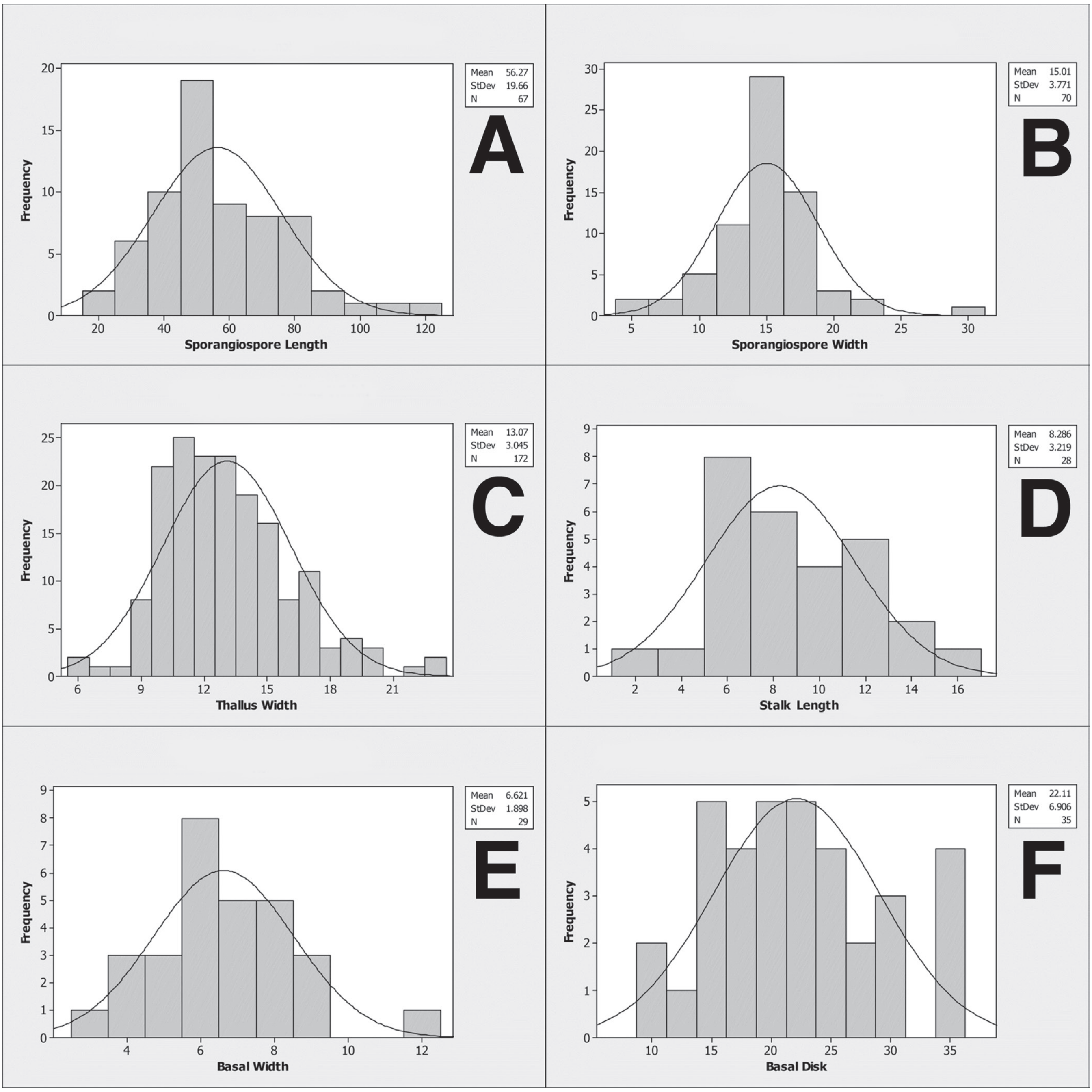

Fig. 2. Frequency plots of key-structure measurements (in µm) in *Enterobryus luteovirgatus*. Sporangiospores: (**A**) length, (**B**) width; (**C**) Thallus width; Holdfast: (**D**) stalk length, (**E**) stalk width, (**F**) basal disk width.

mally distributed: (iv) sporangiospores length ($p = 0.02$), (v) sporangiospores width ($p < 0.005$) and (vii) thallus width ($p < 0.005$). Sporangiospore length (Fig. 2A) has a mean of 56.27 ± 19.66 µm with a range 20–116 µm in the sample (N = 67), where the median of 53 is more representative for the sample. Similarly, sporangiospore width (Fig. 2B) has a mean of 15.01 ± 3.77 µm with a range 6–31 µm in the sample (N = 70), where the median coincides with the mean as expected for a normal distribution. Thallus width (Fig. 2C) values have a mean of 13.07 ± 3.04 µm with a range 6–23 µm, where the median coincides with the mean (N = 172). Thallus length (graph not shown) is extremely variable with values up to 2.1 mm and thus, not a consistent character in *E. luteovirgatus* population. Holdfast characters (Fig. 2D–F) are separated in three components: (i) stalk length, (ii) stalk width and (iii) basal disk width. Stalk length mean is 8.26 ± 3.21 µm with a range 2–15 µm and a median of 7.5 µm (N = 28). Stalk width mean is 6.62 ± 1.89 µm with a range 3–12 µm and a median of 6 µm (N = 29). Finally, the basal disk width is the most variable character with mean 22.11 ± 6.91 µm in a range 10–35 µm (N = 35) and a very close median value of 22 µm although the character values did not follow a normal distribution (p = 0.525).

DISCUSSION

Enterobryus has been considered a problematic taxon, because of the difficulty in establishing reliable characters unique to species due to great intraspecific variation (Litchwardt *et al.* 2001). Rarely, *Enterobryus* species present one thallus and sporangiospore type such as in the case of *E. luteovirgatus*. Most species have a primary and secondary infestation sporangiospore type although not always available in the collected material. In our case we have observed many specimens over the last two years and have only detected one sporangiospore type congruent with the secondary infestation type A. *Enterobryus luteovirgatus* infestation was observed in the hindguts of 93% of all dissected specimens of *A. monilicornis*, which suggests that there is truly one thallus and spore type. Morphological characteristics from *E. luteovirgatus* are not particularly different from other *Enterobryus* species, but the combination of character sizes makes it unique. In comparison with the other 24 *Enterobryus* described species in the online electronic key Lucid (Lichtwardt 2004), the character combination and sizes are sufficient to separate the new species. *Enterobryus luteovirgatus* has similarities with *E. borirae, E. euryuri* and *E. viequensis*. *Enterobryus borirae* has longer (10–12 µm) holdfast stalks longitudinally striated and sporangiospores of types A, G, H and I (Lichtwardt 1958) while *E. luteovirgatus* has only sporangiospores type A. *Enterobryus euryuri* has different holdfast shape (unevenly cylindrical with or without basal disk vs. slightly convex with basal disk), sporangiospores type A, B, C and E with basal swelling in type A sporangiospores (Lichtwardt 1954) not observed in *E. luteovirgatus*. Finally, *E. viequensis* is morphologically very similar to *E. luteovirgatus*, and even their hosts belong to the same genus, *A. arboreus* and *A. monilicornis*, respectively. *Enterobryus viequensis* has sporangiospores type A, E and F and presents a swollen thallus base, which is distinctive of the species and easily separates it from *E. luteovirgatus* (Hernandez Roa *et al.* 2009).

Measurements of characters were statistically analyzed to study their distribution and stability in order to evaluate their usefulness as species descriptors. Traditionally, the mean of the values and their maximum and minimum ranges are reported for each character without taking into consideration the true distribution of these values in the population. Our results (Fig. 2) show that the measurements of almost all characters in *E. luteovirgatus* follow a unimodal distribution, which makes it easier to estimate the representative parameters of the species or population. Unimodality gives an idea of how scattered and concentrated are the values of the parameters in the population, allowing us to understand the variation of the character under study. For example, the sporangiospore length and width show low standard deviation (Fig. 2A–B) indicating that the population values for those characters are close to the mean value and thus, less prone to extreme values. The graphs of both characters present an unimodal distribution, and depicting a tendency of values between certain ranges within the population. On the contrary, the holdfast basal disk width values depart from normality and exhibit a non-unimodal distribution (Fig. 2F). Since the basal disk is a secreted substance from the thallus, its shape and dimensions depend on external factors of the environment (the gut) and are not controlled by the microorganism. Thus, it makes sense that a cementing structure, that is not a perfect circle, differs in area values and has such variation making it an inconsistent character to use in species descriptions. Standard deviation allows for prediction of the reliability of key characteristics measured in function of the population of *Enterobryus* species. Nonetheless, overall morphology (e.g. holdfast shape, relationship of complex structures to one another) is still valuable in itself and should not be underscored by other types of data. This study presents the first description that considers the sampling distribution of character data; therefore it should be seen as a new point of view into the description of *Enterobryus* species.

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