

A NEW SPECIES OF THE GENUS *BUNNYA* H. B. BAKER, 1942
(HELICOIDEA) FROM MÉXICO

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ABSTRACT

We describe a new helicoidean semi-slug based on morphological and molecular evidence. The new species belongs to the genus *Bunnya* and is described from a small agricultural area in Zinacantepec, San Juan de las Huertas, México. The genus *Bunnya* is externally similar to *Xanthonyx*, another Helicoidea genus; both genera have similar shell and body form, and both have a tail horn. Internally, *Bunnya* is similar to *Humboldtiana*, since both have dart-sacs (3 in *Bunnya*, 3–4 in *Humboldtiana*) surrounding the vagina, two dart-bulbs associated with each dart sac, and a gland number similar to the dart-sacs number. Four adult specimens of *Bunnya metli* n. sp. were dissected and compared with the two described species: *B. bernardinae* from Cuajimalpa, México City, and *B. naranjoe* from Sierra de Manantlan, Jalisco. *Bunnya metli* n. sp. is characterized by: very closely-spaced radial riblets (about 23 per mm) crossed by fine spiral wavy threads on the embryonic whorls; unfused glands inserted on the vagina above the dart-sacs and only one dart per sac; a short, swollen penis with a large spherical verge about half the penis size; an elongated bursa copulatrix with a slight constriction in the middle; a long bursa copulatrix duct; and dart sacs with a muscular pad within the vaginal lumen, surrounding the basal part of the genitalia. We performed a phylogenetic analyses using data from fragments of 16S rRNA mtDNA and 28S rRNA genes from one of the collected specimens, as well as other helicoidean sequences from GenBank. Our analyses support the membership of *Bunnya metli* n. sp. in Xanthonychidae. This suggests that the taxonomic position of *Bunnya* in Humboldtianidae or Xanthonychidae warrants reevaluation.

Key words: semi-slug, biodiversity, Helicoidea, México, 16S rRNA mtDNA, 28S rRNA.

INTRODUCTION

The superfamily Helicoidea is a large and diverse group of pulmonates (*sensu* Bouchet & Rocroi, 2005) that includes land snails and semi-slugs with an almost worldwide distribution. The composition of this superfamily has been subject to many changes. No agreement has been reached to define which families should be included, whether it should be regarded as a superfamily, or if it should be divided into various superfamilies (Wade et al., 2007). Besides this, many phylogenetic studies with molecular markers in Helicoidea have been published (Wade et al., 2001, 2007; Steinke et al., 2004; Koene & Schulenburg, 2005; Manganelli et al., 2005; Davison et al., 2005), without consensus between molecular analyses and morphology (Nordsieck, 2013). The most recent classification based on genital

morphology and molecular markers includes 11 of the 19 families proposed by Bouchet & Rocroi (2005), Humboldtianidae among them (Razkin et al., 2015).

The family Humboldtianidae includes snail and semi-slug species, and is defined by the presence of a diverticulum in the bursa copulatrix duct, although sometimes this may be missing; 3–4 dart sacs and same number of glands; glands inserted on vagina above dart sacs; two darts per dart sac (Nordsieck, 2013). This family includes two genera: *Humboldtiana* Von Ihering, 1892, a genus of landsnails consisting of approximately fifty species, distributed from southern Texas to central México in rocky habitats (Thompson, 2006) and *Bunnya*, a genus of semi-slugs, distributed in parts of the Trans-Mexican Volcanic Belt and a part of Durango, and occurring principally in rocky habitats with temperate climates.

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The genus *Bunnya* comprises two species. The first species described was *B. bernardinae* H. B. Baker, 1942, from El Desierto de los Leones, México City. This species was later collected in Temascaltepec, México State (Miller, 1987). The second described species was *B. naranjoae* Miller, 1987, from Sierra de Manantlan Jalisco, Durango and Nayarit (Miller, 1987).

Based on molecular and morphological evidence, we describe a new species of *Bunnya* from Zinacantepec, Toluca, México. Field observations suggest that this species is restricted to maguey plants from family Agavaceae (*Agave americana* L.), a common component of the Mexican landscape, in a small geographic range in the center of México threatened by intensive agricultural practices.

MATERIAL AND METHODS

Four adult semi-slugs were hand-collected on 19 August 2012, and three juveniles were collected on 22 October 2015, all from Zinacantepec, Toluca, México. The juveniles were reared in a terrarium; however, they died before reaching adulthood. The four wild-collected adults from Zinacantepec were drowned in water overnight and then fixed in 70% alcohol. Three of these adults were dissected under a stereomicroscope. Jaws and radula were obtained by soaking them in household bleach and washing them with water after approximately 1 min. Both jaws and radula were micrographed with a scanning electron microscope (JEOL JSM-7800F, Akishima, Tokyo Japan).

The shells were cleaned and three measurements were recorded: shell width (*W*, greatest distance transverse to the shell axis), shell height (*H*, distance from the apex to the basal lip parallel to the shell axis), and aperture width (*A*, distance from the edge of the outer lip to the inner edge of the columellar lip transverse to the shell axis). Shell whorls were counted and photographed, using a scanning electron microscope (Zeiss Sigma VP, Germany).

Specimens were deposited in the Colección Nacional de Moluscos (CNM) of the Instituto de Biología, Universidad Nacional Autónoma de México (México City, México).

Molecular Analysis

DNA was extracted from a fragment of the foot muscle using the DNeasy Tissue kit (QIA-

GEN, Valencia, California, U.S.A.), following the manufacturer's instructions. The region of the 16S rRNA mtDNA gene was amplified with primers 16Sar and 16Sbr (Palumbi, 1996) and another region of the 28S rRNA gene with primers C1 and D2 (Hassouna et al., 1984). The PCR reaction mixtures for both genes were as follows: on 25 µl reaction containing 2.5 µl of 10X PCR buffer, 2.5 mM MgCl₂, 10 mM of each deoxynucleotide triphosphate (dNTP), 13 pmol of each primer, 1U/µL Taq DNA polymerase (Invitrogen, CA, USA) and 2 µl of template DNA. The cycling parameters were: preheating of samples for 5 min at 95°C; 30 cycles of 30 s at 94°C, 50°C for 30 s (58°C, 60 s for 28S), 72°C for 45 s; and a final extension of 7 min at 72°C. PCR amplifications were performed using a Techne TC-5000 Thermalcycler (Burlington, New Jersey, U.S.A.). Amplicons were purified with the PCR product purification kit (Jena Bioscience, Germany), sequenced by Macrogen Inc., Korea, and sequences deposited in the GenBank (accession number for 16S KY886472, and 28S MG000150).

Phylogenetic Analyses

Sequences were manually edited and each gene aligned independently with reference sequences obtained from GenBank using Seaview ver. 4.0 (Gouy et al., 2010). A total of 134 helicoidean sequences, representing 12 families were used: Bradybaenidae, Camaeniidae, Canariellidae, Elonidae, Geomitridae, Helicidae, Helicodontidae, Humboldtianidae, Hygromiidae, Sphincterochilidae, Trissexodontidae and Xanthonychidae (Supplementary material table S1). Other sequences of genus *Bunnya* were not included, because there are not deposited in GenBank. Both alignments were combined or concatenated for the phylogenetic analyses for a final fragment of 649 bp. The most appropriated DNA nucleotide substitution model (GTR) for the data set was determined using the Corrected Akaike Information Criterion (AICc) and the Bayesian Information Criterion (BIC) tests, as implemented in the software jModeltest ver. 2.1.7 (Posada, 2008). Subsequently, we performed phylogenetic analyses employing Maximum Likelihood (ML) with the algorithm implemented in the program PhyML ver. 3.0 (Guindon et al., 2010). The support of the clusters was evaluated with a bootstrap test after 1,000 pseudoreplicates.

SYSTEMATICS

Superfamily Helicoidea
Rafinesque, 1815

Family Humboldtianidae
Pilsbry, 1939

Bunnyinae H. Nordsieck 1987

Genus *Bunnya*
H. B. Baker, 1942

Bunnya metli n. sp.
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2,961 m, 19-Aug-2012 on maguey (*Agave americana* L.), V. Araiza coll. (CNM 6791).

Paratypes: México, Zinacantepec, San Juan de las Huertas, 19°13'58.4"N, 99°45'45.1"W, 2,961m, 19-Aug-2012, 3 specimens on maguey, V. Araiza coll. (CNM 6792, CNM 6793, CNM 6794).

Other Material Examined

From type locality, 22-Oct-2015, 3 specimens on maguey, V. Araiza, M. Becerril and O. Valerio coll. (CNM 6795).

Diagnosis

Animal slug-like. Embryonic whorls with 23 longitudinal riblets per mm crossed by fine spiral wavy threads. Penis short and swollen with a large spherical and smooth verge. Epiphallus

Type Material

Holotype: México, Zinacantepec, San Juan de las Huertas, 19°13'58.4"N, 99°45'45.1"W,

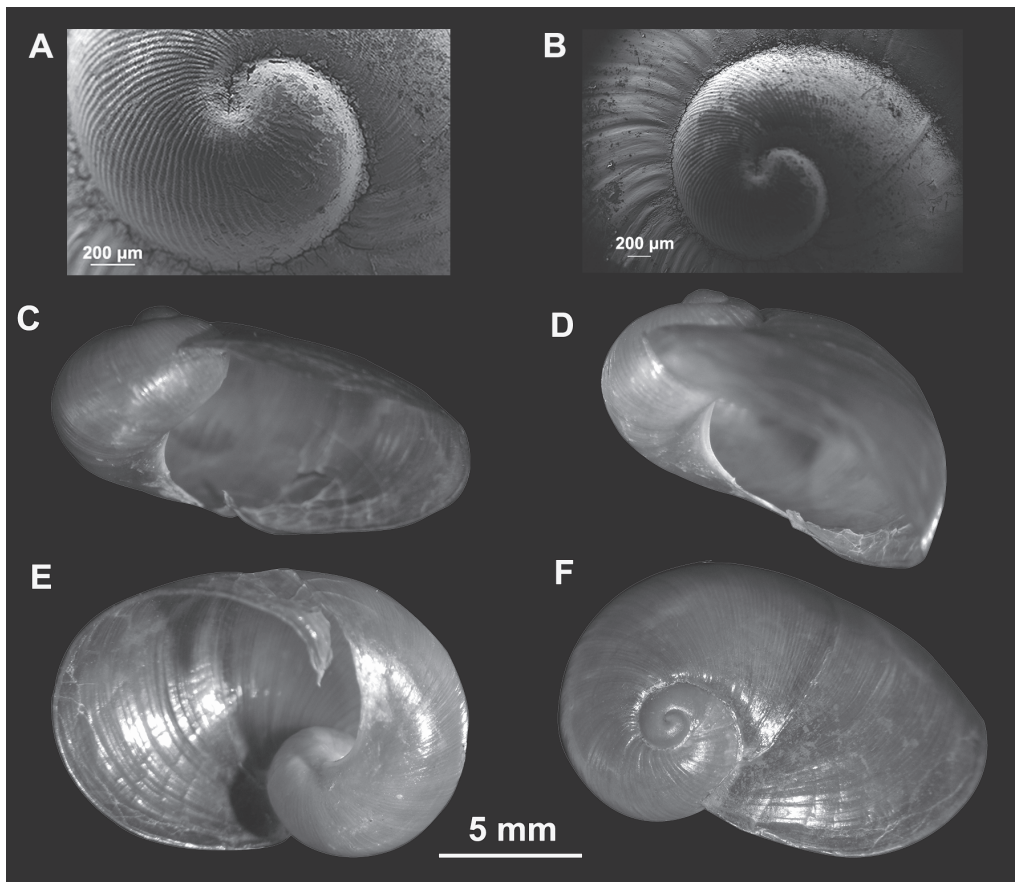


FIG. 1. *Bunnya metli* n. sp. Holotype shell. A: SEM micrograph of the embryonic whorls sculpture; B: SEM micrograph of the body whorl sculpture; C: Apertural view; D: Profile view; E: Ventral view; F: Apical view.



FIG. 2. External appearance of *Bunnya metli* n. sp. shown dorsally (live specimen) and laterally (dead specimen). Scale bar = 10 mm.

long with a flagellum longer than the epiphallus, internally with 3 pilasters. Bursa copulatrix is an elongated sac with a mild constriction in the middle. Three dart sacs, each with a muscular pad located within the vaginal lumen.

Description

Shell (Fig. 1): Imperforate, thin, fragile, glossy, amber, with $2\frac{1}{3}$ whorls, depressed, rapidly increasing in size. Embryonic whorls $1\frac{1}{4}$, first $\frac{1}{4}$ of whorl almost smooth, then sculptured with about 23 radial, parallel and slightly arcuated riblets per mm (Fig. 1A), crossed by very fine and inconspicuous wavy threads. Body whorl with deep growth lines (11 per mm) (Fig. 1B).

Aperture large, ovoid, oblique, wider than high (Fig. 1C). Peristome thin, very fragile, straight. Parietal callus thin, white, inconspicuous. Body whorl suture well impressed,

straight but rising towards the spire making the aperture wider (Fig. 1D). Spire depressed, dome-shaped. Body whorl rounded.

The shell has two layers, a very thin corneous external layer or periostracum that gives the shell its color, this layer reaches the edge except at the columella. The internal layer is shiny, slightly pearly and thicker, it does not expand beyond the corneous layer at the shell edge.

Holotype shell diameter: 13.5 mm; height: 10.0 mm.

External morphology (Fig. 2): Holotype CNM 6791 foot length 41 mm; paratypes CNM 6792 33 mm, CNM 6793 36 mm and CNM 6793 32 mm. Adults cannot withdraw into the shell, foot tapering at the end. Foot holopod. Caudal horn prominent, still visible in dead specimens. Caudal horn without mucus pore. Sole color pattern consisting of five stripes, outermost stripes white ivory,

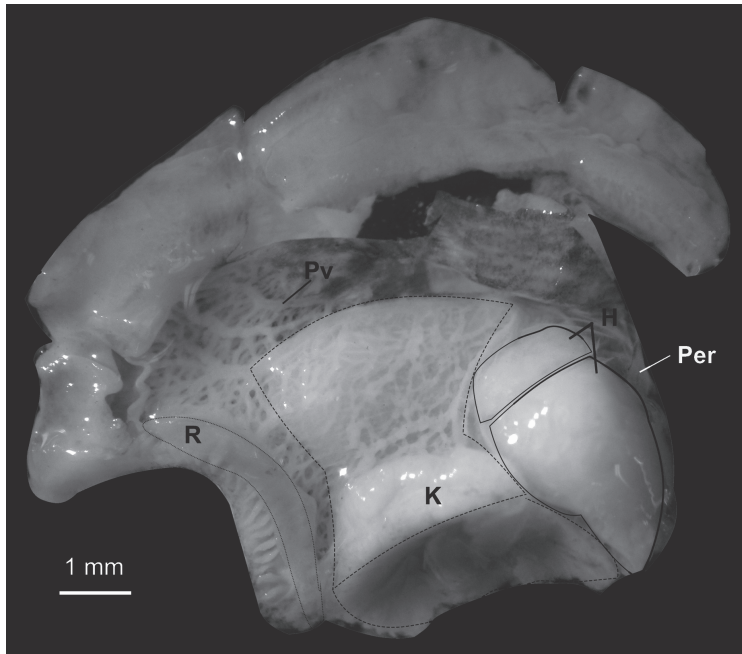


FIG. 3. Pallial organs of *Bunnya metli* n. sp. Abbreviations: H - heart; K - kidney, Per - pericardium, Pv - pulmonary vein, R - rectum.

intermediate stripes grey, middle stripe light grey. Body sculpture: cephalopodium front part with few folds, cephalopodium back part with many transverse folds and some longitudinal deep wrinkles. Posterior lateral foot background ivory with semi round black spots, under the shell spots become more regular in size and shape.

Background color of anterior lateral foot ivory with black blotches forming a longitudinal band. Head background color ivory with a wide dorsomedial fringe grey color. Ocular tentacles grayish becoming darker at the tip. Labial tentacles color uniformly ivory. Caudal horn dorsal and lateral surfaces ivory color, size and shape variable according with snail's activity. Mantle expanded externally over the shell in a continuous lobe, covering about half of the shell, but leaving the spire and part of the body whorl exposed. Mantle covered with prominent papillae. Papillae varying in size and shape, pigmented with semi round black spots, more protuberant in the posterior part. Pallial organs (Fig. 3): Kidney hourglass-shaped, occupying half the mantle cavity. Heart about the same width and length as kidney, auricle and ventricle well developed.

Pulmonary vein highly branched towards the anus and over the kidney.

Reproductive system (Fig. 4): Penis swollen (holotype: length 2.5 mm, width 1.5 mm), inner wall with four longitudinal thin folds, few transversal lamellae, present only between three of the four folds (Fig. 4B). Verge spherical, large with lateral aperture (holotype: diameter, 2.5 mm). Epiphallus long (holotype: length 5.0 mm, width 1.8 mm), separated from the penis by a constriction, inner wall with three longitudinal thick pilasters with transversal grooves in its whole extension (Fig. 4B). Epiphallic flagellum curved, longer than the epiphallus (holotype: length 6.0 mm, width 0.7 mm), inner wall with few longitudinal folds at the beginning and just one fold in the rest of the flagellum. Penial retractor muscle originates on diaphragm, goes under both ocular tentacles and attaches to the middle of the penis. Vas deferens: thin, enters in the epiphallus sub-apically, attached at penis' base by filaments, free thereafter until entering into the prostate base. Vagina: tubular (holotype: length 3.0 mm), thick muscular wall, smooth inside. Three large dart sacs (holotype: length 2.1 mm, width 2.0 mm),

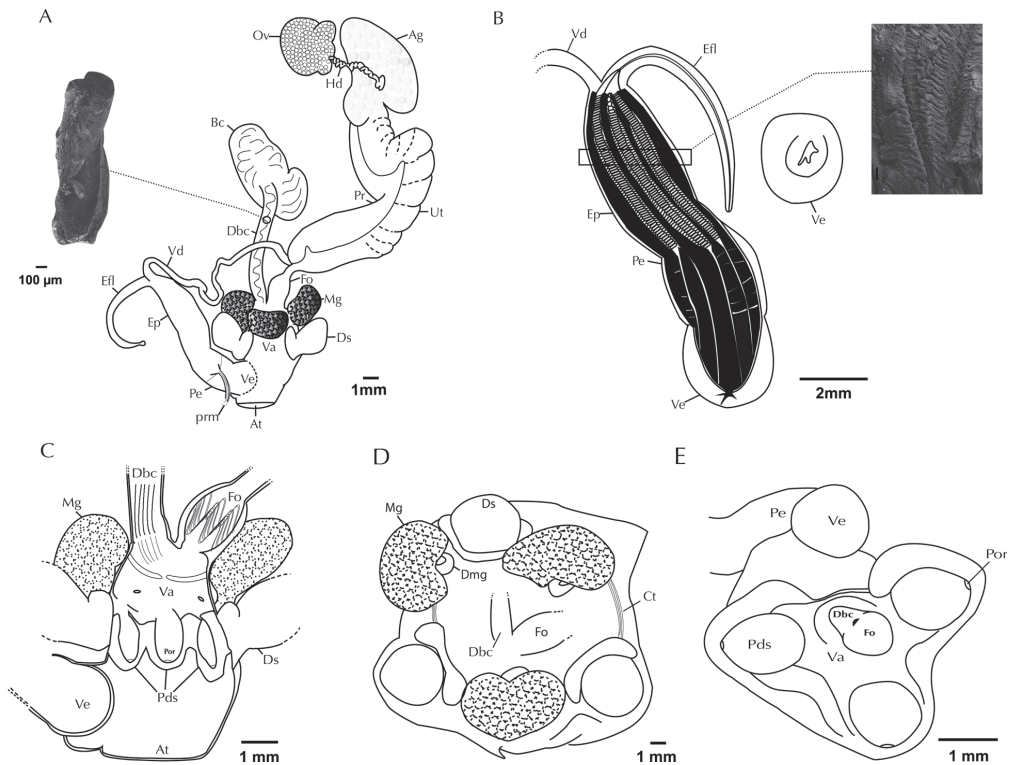
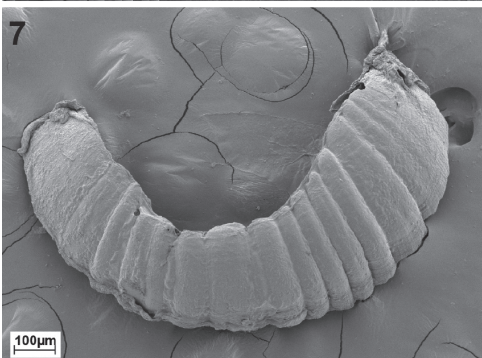
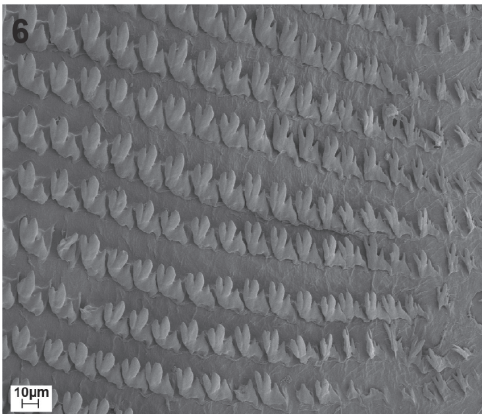
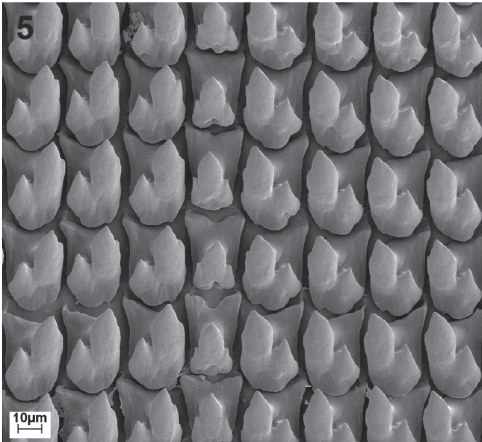


FIG. 4. Reproductive system of *Bunnya metli* n. sp. A: Genital anatomy showing in SEM micrograph a fragment of the spermatophore; B: Verge and penis internal anatomy with SEM micrograph of the inner wall of epiphallus; C: Internal anatomy of the atrium and the vagina; D: External superior view of the mucus glands and dart sacs arrangement; E: Internal ventral view of the mucus glands and dart sacs arrangement. Abbreviations: Ag - albumen gland; At - atrium; Bc - bursa copulatrix; Ct - connective tissue between dart sacs; Dbc - bursa copulatrix duct; Dgm - duct of the mucus gland; Ds - dart sac; Ep - epiphallus; Efl - epiphallus flagellum; Fo - free oviduct; Mg - mucus gland; Hd - hermaphroditic duct; Ov - ovovestis; Pds - protuberance of dart sac; Pe - penis; Por - pore of the dart sac; prm - penis retractor muscle; Ut - uterus; Va - vagina; Vd - vas deferens; Ve - verge.

each one consisting of a compact globular mass that contains the dart and a bilobed transverse muscular sac or dart-bulbs according to Thompson & Brewer (2000) nomenclature, above the main mass (holotype: length 2.5 mm, width 0.5 mm). Dart sacs thick walled. Each dart sac linked to the others at the base of the bilobed sac by a ring of connective tissue (Fig. 4D). Dart sacs with an inner muscular pad in the vagina with a pore and a thick border in the inferior part. In a longitudinal section (Fig. 4C) inside the vaginal lumen, they look like a ribbon of oval pads with a pore towards the atrium, but viewed ventrally, they look like pads around the free oviduct and bursa copulatrix entrance; the free oviduct and bursa copulatrix entrance

share a spinther (Fig. 4E). The three dissected specimens each had only one dart per sac. Three saccular mucus glands (holotype: length 3.2 mm, width 1.5 mm) above the dart sacs, diagonally to them, surrounding the upper section of the vagina; each gland entering into the vagina through its own duct. Bursa copulatrix (holotype: length 6.4 mm, width 3.1 mm) elongated with a slight constriction in the middle, inner wall with many longitudinal folds. Bursa copulatrix duct moderately wide (holotype: length 6.9 mm, width 0.8 mm), inner wall with six very thin longitudinal folds, this duct enters into the vagina by a single sphincter shared with the free oviduct. The three specimens dissected each had a long, helical horny spermatophore rather straight

in the duct and coiled in the bursa copulatrix (Fig. 4A), visible through the surface. Free oviduct very short (holotype: length 3.0 mm, width 1.5–0.7 mm), covered by connective tissue that attaches the free oviduct strongly



FIGS. 5–7. Radula and jaw. FIG. 5: Central and lateral teeth; FIG. 6: Marginal teeth; FIG. 7: Jaw.

to the vagina and compresses it longitudinally; wider at the base and narrower near the uterus, the free oviduct enters into a little chamber shared with the bursa copulatrix duct. Finally, the shared chamber enters into the vagina through a sphincter inner wall with numerous transversal folds (Fig. 4C). Uterus long, well developed. Prostate adnate to uterus, almost as long as the uterus. Albumen gland short and massive. Talon very short, exposed. Hermaphroditic duct serpentine distally and less convoluted near the albumen gland. Ovotestis a little longer than wide, compact, with numerous acini, embedded in the digestive gland. Right ocular tentacle retractor passing between the reproductive organs.

Radula and Jaw: A ribbon with an average of 63 rows of teeth. Tooth formula C+13+16-19. Central tooth distinctly smaller than laterals, slightly triangular mesocone with a small ectocone at each side (Fig. 5). Lateral teeth bicuspid, mesocone with a vestigial endocone forming a superior lateral notch, ectocone smaller than mesocone both sharply-pointed (Fig. 5). Approximately thirteen marginal tricuspid teeth, mesocone and ectocone rounded, endocone pointed. Outer marginal teeth very variable from tricuspid to polycuspid (Fig. 6). Jaw arched with 15 ribs (Fig. 7).

Type Locality

México, Toluca, Zinacantepec, San Juan de las Huertas on wild maguey (*Agave americana* L.) at the edge of corn crops. 19°13'58.4"N, 99°45'45.1"W, 2,961 m.

Habitat

Specimens were collected in an agricultural town. Its predominant climate is semi-cold (9°C–17°C), sub-humid with summer rain. The adults were found under rotten maguey leaves in August, whereas juveniles were found on the maguey plant, between the lower leaves, in October. The maguey plants were surrounded by maize crops during the first visit and potato crops during the second visit.

Etymology

The species name *metli* (*metl* = maguey) is from Náhuatl, a native Mexican language. The latinized word takes the genitive form, meaning “from maguey”, the plant on which the specimens were collected.

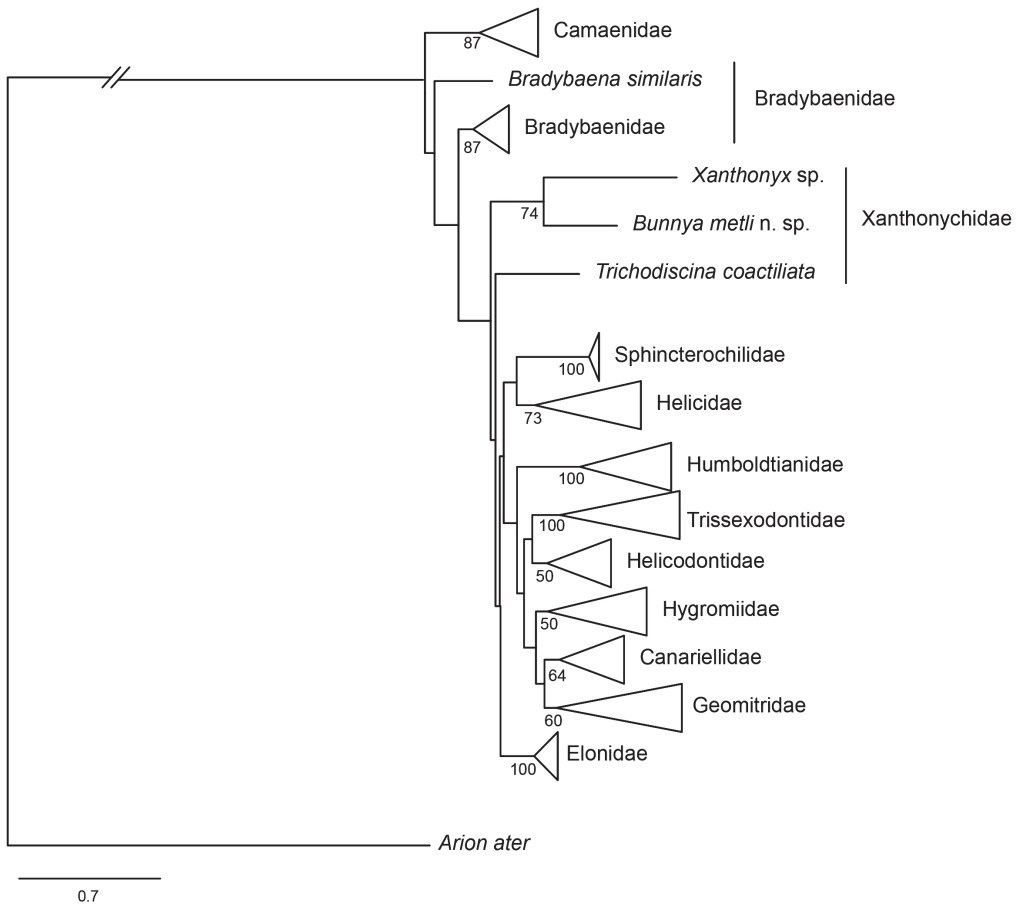


FIG. 8. Phylogenetic tree inferred using the Maximum Likelihood method using 16S rRNA mtDNA and 28S rRNA sequences. Sequence of *Arion ater* was included as outgroup. Bootstrap values after 1,000 pseudoreplicates are shown at nodes. Scale bar = number of substitutions per site.

Molecular Analysis

Phylogenetic analyses recovered 12 groups (families), which were well-supported (bootstrap > 50%) and that belong to superfamily Helicoidea *sensu* Bouchet & Rocroi (2005). *Bunnya metli* n. sp. was associated with *Xanthonyx* sp.

Comparisons

The embryonic whorl sculpture of *Bunnya metli* n. sp. differs from that of *B. bernardiniae* and *B. naranjoae* by having more radial riblets per mm, 23 compared with 14 and 10, respectively. The three species have closely spaced microscopic spiral grooves. In *B. metli*

n. sp., the penis is short and swollen and has a large spherical smooth verge about half the penis size, with a lateral star-shaped aperture, a shape that is formed by the verge's internal longitudinal thin folds. In contrast, in *B. bernardiniae* the verge is cylindrical, wrinkled and papillated and the aperture is demarcated by three flattened papillae at the tip. There are some differences in the nomenclature of these structures: we use the term "verge aperture", whereas Baker (1942) used "epiphallic opening", but according to the figures we refer to the same structure. Miller (1987) does not mention the presence of a verge in *B. naranjoae*. Moreover, in *B. metli* the epiphallus is long and has a flagellum that is longer than the epiphallus with three internal pilasters. Likewise, in *B.*

bernardinae, the epiphallus is long, but the flagellum is short and has four internal pilasters. In *B. naranjoe*, the epiphallus is coiled and has five or six pilasters. The bursa copulatrix is an elongated sac with a mild constriction in the middle, whereas in *B. bernardinae* it is an ovoid sac with a large diverticulum and in *B. naranjoe* it is spherical and has an ear-shaped or bilobed appendix in the apex. Each dart sac of *B. metli* has an internal muscular pad protuberance. These protuberances surround the vagina internally and are absent in the other two species.

In México, the superfamily Helicoidea has five genera of semi-slugs; four belong to the family Xanthonychidae (*Xanthonyx* Crosse & Fischer, 1867; *Metotracon* Pilsbry, 1900; *Semiconchula* Naranjo-García, Polaco & Pearce, 2000, and *Echinix* Thompson & Naranjo-García, 2012) and one to the family Humboldtianidae (*Bunnya* H. B. Baker, 1942) (Nordsieck, 2013).

The genus *Echinix* is a recently described slug-like. This genus has the greatest external differences from *Bunnya*: its size is much larger than any of the previously described semi-slugs, measuring up to 200 mm in length; the shell is discoidal and is completely embedded in the mantle, and the sides of the body and tail are covered with moderate to large, high, conical glandular papillae. Although the mantle in *Bunnya* is covered with prominent papillae, these are not as large and sharp as in *Echinix*. Internally, *Echinix*, has only one dart sac inserted in the vagina, with two dart papillae in its base and two dart glands (Thompson & Naranjo-García, 2012), whereas *Bunnya* has three dart sacs and three mucus glands inserted in the vagina.

The genus *Metotracon* has a totally different shell shape from *Bunnya*, since it is uncoiled, depressed and covered with a thin cuticle. The dart sac in *Metotracon* is ovate and very large inserted on the atrium, while *Bunnya* has three dart sacs. In addition, *Metotracon* possess two well developed elongated mucus glands inserted in dart sac, whereas *Bunnya* has three mucus glands entering into the vagina (Pilsbry, 1900).

Externally, the genus *Bunnya* looks more like *Xanthonyx*; both have a caudal horn and a shell partially covered by the mantle, a largely exposed spire and a very similar embryonic whorl sculpture. Nevertheless, they differ internally; *Xanthonyx* has one small dart sac, while *Bunnya* has three. Also, the mucus glands differ in shape and size. In both genera, the mucus gland insertions are located in the vagina,

however, *Xanthonyx* has two large, oblong mucus glands while *Bunnya* has three small and saccular mucus glands.

The genus *Semiconchula* differs from *Bunnya* as the shell is entirely concealed by the mantle; it also has no caudal horn, the jaw has none or only two ribs; and it possesses two mucus glands, one massive and one reduced. Depending on the species, the mucus glands enter into the atrial sac, either by one or by two mucus gland ducts (Naranjo-García et al., 2000; Naranjo-García, 2003).

The phylogenetic tree suggests that *Bunnya metli* belongs to Helicoidea, but not to family Humboldtianidae, as it was suggested by Bouchet & Rocroit (2005), Schileyko (2004) and Nordsieck (2013) (Fig. 8), despite of *Bunnya* spp. share the disposition of dart sacs and mucus gland that around the vagina, which is a characteristic of this family. This discrepancy between the molecular phylogeny and the morphological classification should be taken with caution, because it is necessary deepen in molecular studies. The poor conservation of *Bunnya* specimens available in foreign collections (Mejía & Zúñiga, 2007), and the absence of sequences in GenBank exacerbate the difficulty of phylogenetic molecular comparison between *Bunnya* species and other Helicoidea. This situation would be improved by including more localities and specimens of *Bunnya* spp., other Helicoidea, as well other genes, in order to increase our understanding of the phylogenetic relationships among Helicoidea, particularly those from México.

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