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MOLECULAR AND MORPHOLOGICAL TOOLS TO DISTINGUISH *SCYPHOPHORUS ACUPUNCTATUS* GYLLENHAL, 1838 (CURCULIONIDAE: DRYOPHTHORINAE): A NEW WEEVIL PEST OF THE ENDANGERED CENTURY PLANT, *AGAVE EGGERSIANA* FROM ST. CROIX, U.S. VIRGIN ISLANDS

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Abstract.—The agave snout weevil (AGW) or sisal weevil, *Scyphophorus acupunctatus* Gyllenhal is here reported for the first time in St. Croix, U.S. Virgin Islands (USVI) where it threatens *Agave eggersiana* Trel., a USVI endemic and endangered century-plant. We provide molecular, morphological, and behavioral characters to successfully distinguish the two known *Scyphophorus* species at all developmental stages. We identified seven new larval characters on the mandibles and characters relating to the chaetotaxy of the labrum and labio-maxillary complex as well as new, putatively informative characters for weevil systematics: chitinized arm of mentum (postlabial strut or postlabial bracon) and the presence of 4 ventral malar setae, instead of 5. In the pupae, the difference in number and placement of rostral setae were also found to be diagnostic. We analyzed two genes, mtCO1 and EF1a, to confirm the identity of the immatures. Phylogenetic analysis of both genes separately and together suggests a clear pattern of substantial phylogeographic structure with specimens clustering by geographic location and this pattern strongly suggests the presence of cryptic species or allopatrically diverged populations. We provide management recommendations for the protection of *Agave eggersiana* against the threat posed by ASW. We also report, for the first time, the presence of *Sphenophorus cubensis* (Buchanan) in St. Croix and *Scyphophorus yuccae* Horn in Panama on *Hesperoyucca whipplei* (Torr.) Trel. (Asparagaceae: Agavoideae).

Key Words: larval morphology, Palm weevils, COI, EF1a, conservation, Endangered Species Act, West Indies, *Scyphophorus yuccae*, *Sphenophorus cubensis*

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The Agave Snout Weevil (ASW) or Sisal Weevil, *Scyphophorus acupunctatus* Gyllenhal, 1838 is one of the most destructive pests of cultivated agave, where it can destroy up to 70% of commercial crops, costing millions of dollars in damage to global agro-industries including tequila, mezcal, perfume, henequen, nardo, pulque, and fiber manufacturing (Camino Lavin et al. 2002). From its native range in the SW United States and Mexico, it has tracked its host plant and invaded regions of Australia, Belize, British Virgin Islands, Cayman Islands, Costa Rica, Cuba, Curaçao, Cyprus, Dominican Republic, Dutch Antilles, El Salvador, France, Guatemala, Greece, Haiti, Honduras, Indonesia, Italy, Jamaica, Kenya, Netherlands, Nicaragua, Saudi Arabia, Spain, Puerto Rico, South Africa, Tanzania, USA (AK, CO, FL, GA, HI, KS, and NV), and the U.S. Virgin Islands (St. John and St. Thomas); (Materu & Hopkinson 1969; Vaurie 1971; Pott 1975; O'Brien and Wibmer 1982; Colombo 2000; Gibney 2004; Flinch and Alonzo-Zarazaga 2007; EPPO 2008; Abbazzi and Maggini 2009; Setliff and Anderson 2011; Vassiliou and Kitsis 2015; CABI 2015). In 2010, *S. acupunctatus* was twice reported anecdotally from north central St. Croix, U.S. Virgin Islands, constituting a potential threat to the endemic endangered century-plant, *Agave eggersiana* Trel. (Asparagaceae: Agavoideae). In St. Croix, *A. eggersiana* has a limited distribution and was thought to be extinct in the wild, but known from cultivation locally (Proctor and Acevedo-Rodríguez 2005). In order to protect *A. eggersiana*, it was listed as endangered in the Territory of the U.S. Virgin Islands (USVI) (DPNR 1991) and after further surveys found naturally occurring populations; the U.S. Fish and Wildlife Service (USFWS) subsequently listed it as endangered in September, 2014 (USFWS 2014a). The presence of *S. acupunctatus* on St. Croix has not previously been confirmed, but was listed as a potential threat to *A. eggersiana* given its detrimental effects on other *Agave* spp. (USFWS 2014a, b).

Scyphophorus acupunctatus has been reported in the nearby islands of St. Thomas, USVI (since 1994), St. John, USVI (2001; various contributors 2015) (Gibney 2004), Tortola (British Virgin Islands, BVI) (2000: Osborne 2002; Gibney 2004), Guana, BVI (Valentine and Ivie 2005), and Puerto Rico (2009: Setliff and Anderson 2011). *Scyphophorus acupunctatus* is believed to have spread to St. Croix, USVI, where it may constitute one of the main threats identified for *Agave eggersiana*. This agave has a restricted distribution on the island where it is found on coastal cliffs and dry coastal shrub lands on a few localities across approximately 24 hectares in eastern St. Croix (Figs 1, 2) (USFWS 2014a, b). The range and population size of *A. eggersiana* have been reduced partly due to habitat alteration, development and poaching associated with the ornamental trade (USFWS 2014a). The risk of extinction of *A. eggersiana* increases with the presence of this weevil in St. Croix, as has been the case on St. John, for the Puerto Rican Bank endemic *A. missionum* Trel. (Gibney 2004).

In December of 2013, the USDA Forest Service's Forest Health Protection Program, in collaboration with the USVI Department of Agriculture (VIDOA), the USVI Department of Planning and Natural Resources (DPNR), the University of the Virgin Islands (UVI), the USDA Animal Plant Health Inspection Service, Plant Protection and Quarantine (USDA APHIS-PPQ), and the USDA Natural Resources and Conservation Service (USDA NRCS) conducted an island-wide ASW survey in St. Croix to confirm the presence of the weevil and to provide

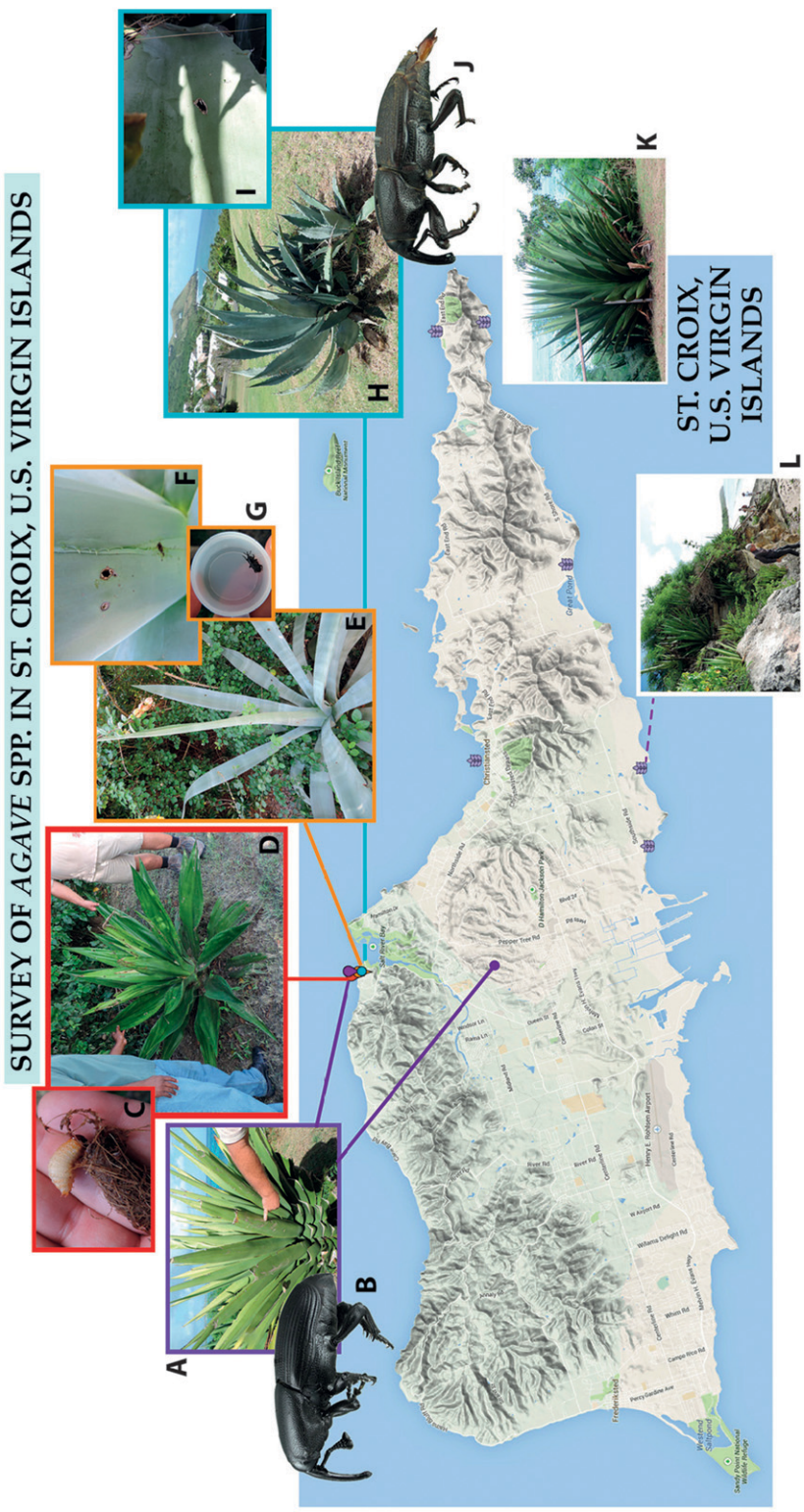

Fig. 1. Map of St. Croix, U.S. Virgin Islands illustrating survey locations where insects were discovered. Location of *Agave eggersiana*'s critical habitat (USFWS 2014b) is denoted by a violet compound leaf; A, *Agave eggersiana* endemic and endangered century plant from the island of St. Croix with William Coles (DPNR) pointing at scars on the leaves caused by an unknown object; B, *Scyphophorus acupunctatus* adult, lateral view; C, *S. acupunctatus* larva (lateral view) and cocoon found on *Furcraea foetida* (L.) Haw. (Asparagaceae) (D); D, *Furcraea foetida* exotic to St. Croix; E, *Agave fourcroydes* Lem., (Asparagaceae) exotic to St. Croix; F, damage by *S. acupunctatus* to *A. fourcroydes*; G, *S. acupunctatus* in cup, found on (E, F), dorsal view; H, *Agave* sp., (Asparagaceae) exotic to St. Croix; I, possibly damage by *Sphenophorus cubensis* (Buchanan); J, *Sphenophorus cubensis*, lateral view, reported for the first time in St. Croix found on (H); K, L, *Agave eggersiana* in critical habitat sites, St. Croix.

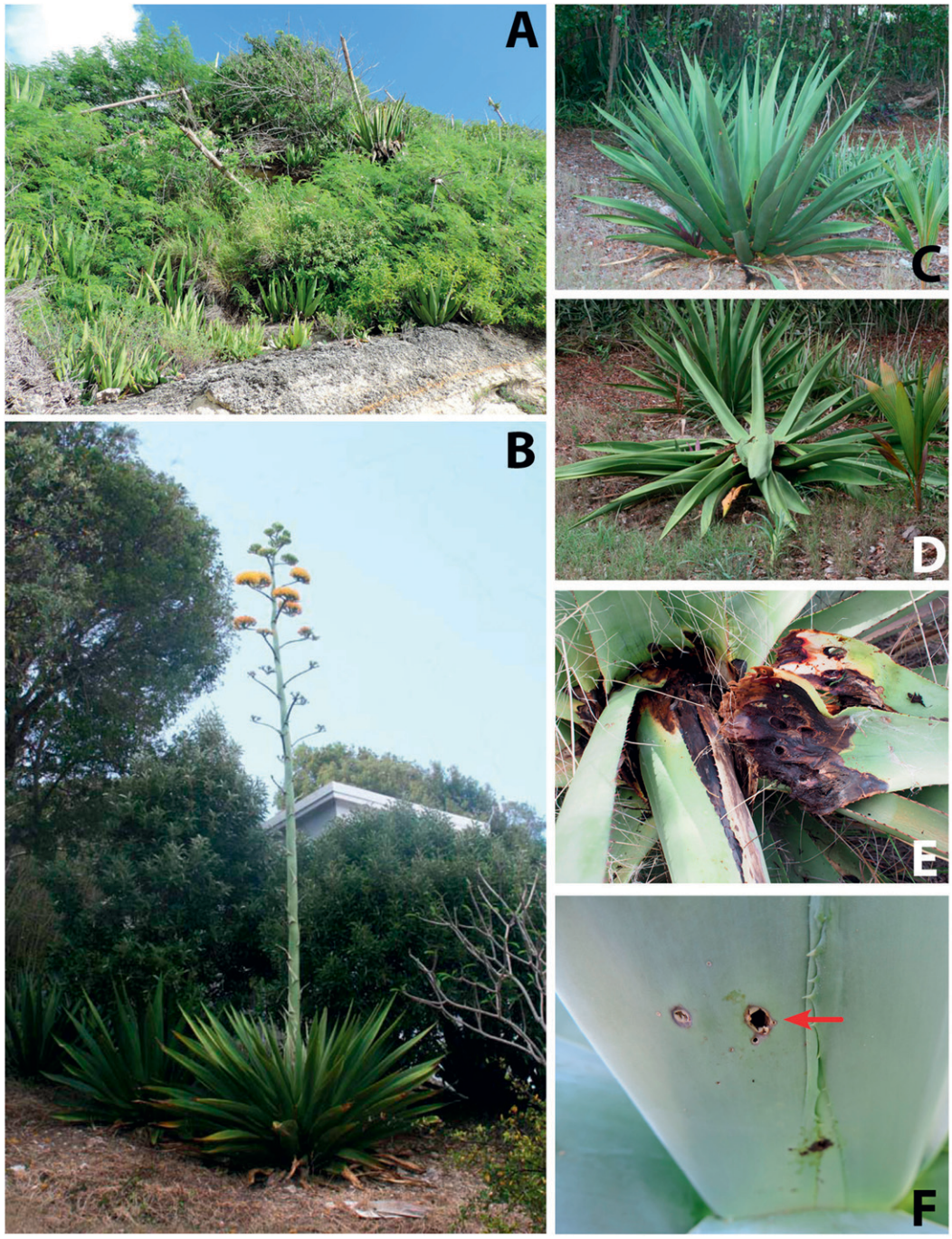

Fig. 2. Habitat and plants. A, Critical habitat of *Agave eggersiana* in St. Croix, USVI; B, *Agave eggersiana* with inflorescence; C, healthy vegetative *Agave eggersiana* in St. Croix; D, same plant of *Agave eggersiana* in "C" dying after presence of *Scyphophorus acupunctatus*; E, decomposing *Agave palmeri*. in Portal, Arizona with visible characteristic oval-shaped feeding damage caused by the tunneling weevil; F, typical dryophthorine feeding/oviposition wound on *Agave fourcroydes*; note the characteristic oval feeding hole surrounded by a darkened ring.

recommendations to the U.S. Fish and Wildlife Service (USFWS), the DPNR, and the VIDOA regarding its potential threat to St. Croix's endemic agave (Figs 1, 2). The specimens collected as part of this survey were tentatively identified as *Scyphophorus acupunctatus* by CWTS and JK and sent to MLCh at the Systematic Entomology Laboratory in Washington, DC for confirmation.

On November 2014, JK, subsequently found six additional adults and four variably sized larvae and one pupa of *S. acupunctatus* at the base of a recently killed, small (approximately 3 feet wide), 3–4 year old *Agave eggersiana* in his yard in Mary's Fancy, central St. Croix (Figs 2B, C, D). By April, 2015 two more *A. eggersiana* in the property showed infestation by immature *S. acupunctatus*, thus confirming the weevil's ability to feed on this plant.

Species identification of adult *Scyphophorus* is relatively simple as there are only two species currently included in the genus: *S. acupunctatus* and the more geographically restricted *Scyphophorus yuccae* Horn, 1873 (or yucca weevil) with confirmed host plant associations with only two species: *Hesperoyucca whipplei* (Torr.) Trel. and *Yucca gloriosa* L. (Vaurie 1971; O'Brien & Wibmer 1982). *Scyphophorus yuccae* has also been reported on *Agave amaniensis* Trel. and Nowell (=*A. sisalana* Perrine) (Maddison and Crosby 2009).

Vaurie (1971) reviewed the genus and identified a suite of diagnostic species- and genus-level characters. In general, they are ovate black weevils (Figs 1B, 10C) reaching a length between 8–24 mm (Vaurie 1971) with minimal sexual dimorphism and few interspecific differences distinguishing *S. acupunctatus* from *S. yuccae*. Of the characters that Vaurie (1971) used to distinguish the two species, the most reliable and easy to observe is the shape of the spongy apex of the antennal club. In *S. acupunctatus* the spongy apex of the antennal club is retracted and concave and not visible in lateral view (Figs 9A, B; 10C); In *S. yuccae*, the spongy apex is truncate and carinate and visible, albeit narrowly, in lateral view (Figs 9C, D). While characters to distinguish the adult forms of the species of *Scyphophorus* were provided (Vaurie 1971, Woodruff and Pierce 1973), there are currently no diagnostic tools to distinguish the immature forms; however Cotton (1924) and Anderson (1948) provided illustrated descriptions and keys to distinguish the larvae of known dryophthorine genera. The key character to distinguish *Scyphophorus* among other genera is the presence of a pair of caudal projections on abdominal segment IX (Figs 3A, B, C).

Biology

The distribution of *Scyphophorus acupunctatus* was summarized by Vaurie (1971) and includes localities in both hemispheres. In North America, *S. acupunctatus* has been found in the southern regions of the U.S. through Central America and into northern parts of South America (Vaurie 1971). Due to its high fecundity and broad host range on plants in the family Asparagaceae, with its highest host association being with plants in the genera *Agave* L. and *Furcraea* Vent. (Woodruff and Pierce 1973), it has spread to regions in East Africa (Kenya and Tanzania) and islands in the Australasian region (Vaurie 1971). *Scyphophorus acupunctatus* has also recently been associated with a new host plant in South Africa, *Agave salmiana* Otto ex Salm-Dyck (Smith et al 2012). Other known host plant associations of *S. acupunctatus* include *Agave americana* L., *A. atrovirens* Karw. Ex Salm-Dyck, *A. attenuata* Salm-Dyck, *A. dasyliriodes* Jacobi and Bouch, *A. lechuguilla* Torr., *A. missionum*, *A. palmeri* Engelm., *A. sisalana, A. shawii*

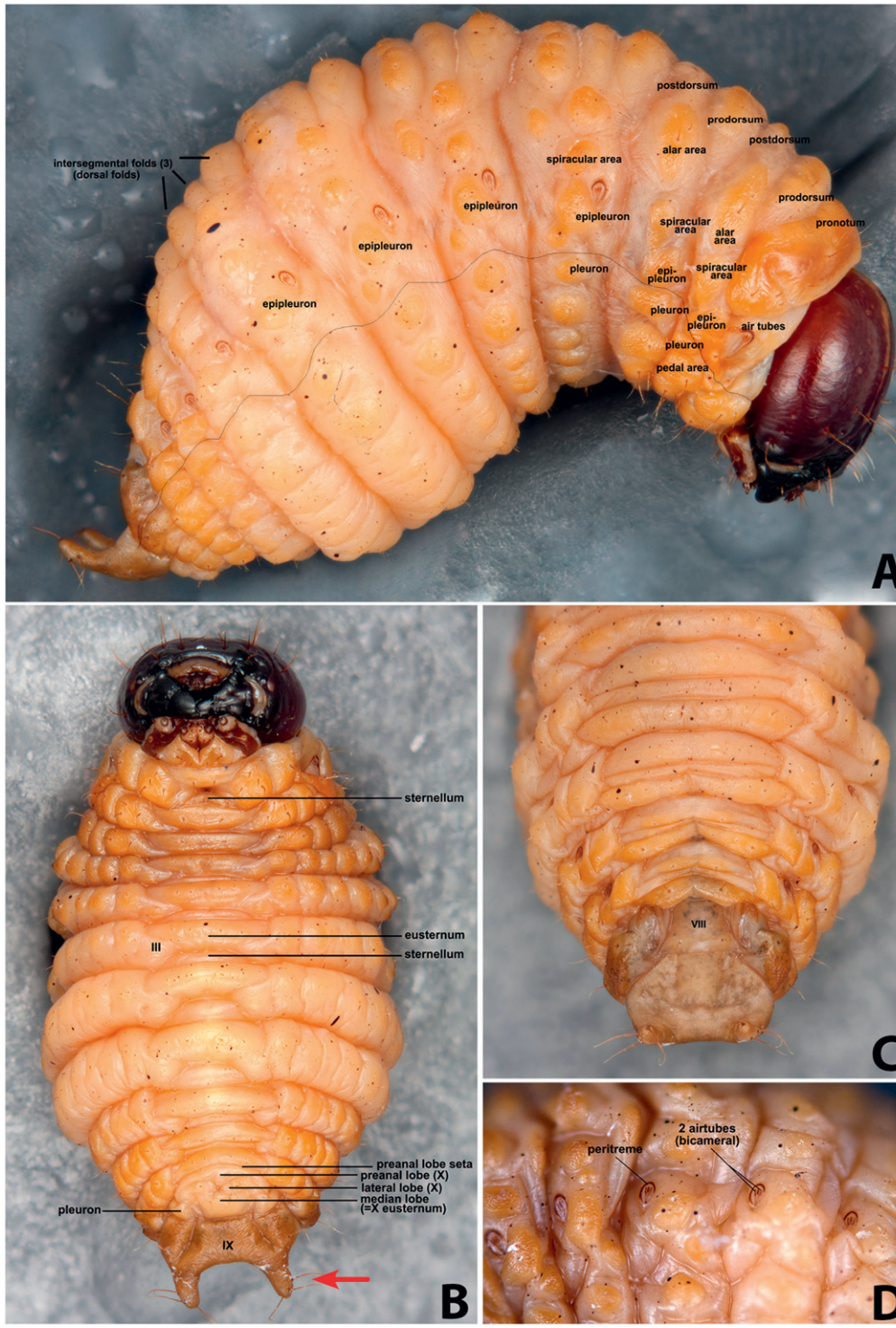

Fig. 3. *Scyphophorus acupunctatus* larva collected in St. Croix. A, lateral view; B, ventral view; C, caudal view; D, detail of spiracles, lateral view.

Engelm., *A. vera-cruz* Mill. (=*A. mexicana* Lam.), *A. victoriae-reginae* T. Moore (=*A. ferdinandi-regis* A. Berger), *Dracaena draco* (L.), *Furcraea hexapetala* (Jacq.) Urb. (=*A. cubensis* Jacq.), *Furcraea tuberosa* (Mill.) Aiton, *Polianthes tuberosa* L., *Sansevieria trifasciata* Prain, and *Yucca glauca* Nutt. (Woodruff and Pierce 1973; Camino Lavin et al. 2002; Gibney 2004; Brown 2011). The other species in the genus, *S. yuccae*, has a more restricted distribution and host association and has been relatively less studied. *Scyphophorus yuccae* was reported by Vaurie (1971) to be found in parts of the Southwestern U.S. (AZ, CA, and NM) and northern Baja California, Mexico and is associated with *Yucca gloriosa* and *Hesperoyucca whipplei* (Vaurie 1971). Adults have also been collected from the flowers of the Joshua tree (*Yucca brevifolia* Engelmann). *Scyphophorus yuccae* is most abundant in California and the distribution mirrors that of its host plants. Reports of this species in Pima County, AZ and Brewster County, TX are possibly incidental records as a result of weevil introductions along with its host plants.

The larvae of *S. acupunctatus* burrow into the apical meristem of the agave rosette where they feed on the inner tissues of the plant. Once the larva is ready to pupate, it burrows towards the roots of the plant to form its cocoon (Smith et al. 2012). In contrast, larvae of *S. yuccae* feed in the base of the inflorescence and move up the stalk of the inflorescence where pupation occurs (Huxman et al. 1997). In both cases the extensive tunnels made by the larvae weaken the plants, making it prone to fire damage and fungal infections (Smith et al. 2012). See Waring (1987) for additional biological information on different patterns of host entry and pupation construction depending on host species.

Scyphophorus acupunctatus is notorious for its ability to infest native and nonnative, blooming and non-blooming plants in Asparagaceae. We present the results of the collaborative survey of St. Croix *Agave* species for the presence of *S. acupunctatus* and provide molecular, morphological, and behavioral characters useful for successfully distinguishing *Scyphophorus* species at all developmental stages.

Materials and Methods

Survey: The survey was conducted visually inspecting the leaves and searching for burrowing holes on every *Agave* and Asparagaceae species observed along the main roads of St. Croix and areas of Protestant Cay, including visiting all the known six populations of *A. eggersiana* (Fig. 1). The *A. eggersiana* individuals planted on Buck Island Reef National Marine Monument were monitored by CWTS on September 25, 2012 and no ASW were observed.

Description and Examination: Slide preparations made by Cotton (1924) and Anderson (1948) housed at National Museum of Natural History, Washington, DC (USNM) as well as additional specimens from the USNM collection were used for comparative morphology. Characters that were studied include chaetotaxy; mouthpart setation; and general external features. The majority of characters are new or were sourced from the following publications: Cotton (1924); Anderson (1948); Wattanapongsiri (1966); Woodruff and Pierce (1973); May (1987, 1993, 1994); Kuschel (1995); Marvaldi and Morrone (2000); Valdés Estrada et al. (2010); Wang et al. (2011); and Lawrence et al. (2011). For description of the pupa the following sources were consulted: Wattanapongsiri (1966); Marvaldi (1997); and Wang et al. (2011). Terminology follows Anderson (1947); May (1994); and Oberprieler et al. (2014).

The World Checklist of Selected Plant Families (WCSP 2015) and The Plant List (2013) were consulted to verify plant names. Species descriptions were facilitated by the program vSysLab: a virtual Systematics Laboratory (Johnson 2010). We based our descriptions on specimens that we can reliably associate, for example when all stages were collected during the same event on the same plant.

All specimens, unless otherwise indicated, are deposited at the USNM. Specimen data was also included from the West Indian Beetle Fauna Collection made available by Dr. Michael Ivie (Montana State University).

Molecular Sequencing: COI and EF1a sequence data were obtained from specimens of *S. acupunctatus* listed in table 1 and referenced in the material examined. These specimens are from *A. eggersiana*, *A. fourcroydes,* and *Furcraea foetida* (L.) Haw. and *A. palmeri* Engelm. and AZ, USVI-St. Croix, Mexico and Guatemala. DNA was extracted from one or two legs or the headless larval body using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). PCR amplification of the DNA barcode region of cytochrome oxidase subunit I (COI) was performed using primers LCO (Folmer et al. 1994) and HeloCO-2198R (5'-TATACTTCTGGATGTCCAAAGAATCA-3'). Elongation factor-1a was amplified using primers EFS149 and EFA1043 (Normark et al. 1999). All PCRs were performed on a Tetrad 2 thermocycler (Bio-Rad, Hercules, CA, USA) with the following "touchdown" program: initial denaturation for 2 min at 92°C, 12 touchdown cycles from 58°C to 46°C (10 s at 92°C, 10 s at 58-46°C, 1 min at 72°C), 27 cycles at 10 s at 92°C, 10 s at 45°C, 1 min at 72°C, and a final extension for 7 min at 72°C. PCR products were enzymatically purified for sequencing using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA). Sequences were generated with the amplifying primers using the BigDye Terminator v3.1 Sequencing kit (Applied Biosystems, Foster City, CA) and fractionated on an ABI 3730XL Genetic Analyzer. Sequences were edited in Geneious R7 (Biomatters, New Zealand). Sequences and specimen records have been deposited in the Barcode of Life Data System

Table 1. Summary of specimens and genes sequenced in this study with information on host plant and developmental stage.

	Scyphophorus acupunctatus Specimen Code	mtDNA protein-coding gene	Nuclear-protein coding gene	Region	Host-plant	Developmental Stage
1	USNMENT01070994	COI	EF1a	AZ	*Agave palmeri*	Adult
2	USNMENT01070995	COI	EF1a	AZ	*Agave palmeri*	Adult
3	USNMENT01070996	COI	EF1a	AZ	*Agave palmeri*	Adult
4	USNMENT01070997	COI		AZ	*Agave palmeri*	Adult
5	USNMENT01070998	COI	EF1a	AZ	*Agave palmeri*	Adult
6	USNMENT01070999	COI	EF1a	AZ	*Agave palmeri*	Adult
7	USNMENT01070993	COI	EF1a	USVI-St. Croix	*Agave fourcroydes*	Adult
8	USNMENT01070992	COI		USVI-St. Croix	*Agave eggersiana*	Adult
9	USNMENT01070990	COI		Guatemala	N/A	Adult
10	USNMENT01070989	COI	EF1a	Mexico	N/A	Adult
11	USNMENT01070988	COI		Guatemala	N/A	Adult
12	USNMENT01070984	COI		USVI-St. Croix	*Furcraea foetida*	Larva
13	USNMENT01075004	COI	EF1a	USVI-St. Croix	*Agave eggersiana*	Adult
14	USNMENT01074904	COI		USVI-St. Croix	*Agave eggersiana*	Adult

(BOLD) and GenBank under the accession numbers KU896919-KU896940. GenBank was mined for sequences for the outgroup *Cosmopolites sordidus* (COI and EF1a); *Scyphophorus yuccae* (COI and EF1a) and 4 COI and 1 EF1a *S. acupunctatus* sequences.

Phylogenetic Analysis: DNA Sequences were managed using Mesquite 3.04 (Maddison and Maddison 2015) and aligned through Mesquite with ClustalX 2.1 (Larkin et al 2007) using default settings under the function "Do Complete Alignment" and subsequently checked by eye. Third codon positions were specified. Three different analyses were undertaken using Mr Bayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003): 1. COI; 2. EF1a and 3. Concatenated COI and EF1a genes. Taxon sampling was reduced for the latter two analyses because despite our best efforts we were unable to amplify EF1a for some specimens. The parameters for the Bayesian analyses are as follow: COI: 20 taxa; 658 base pairs (characters); DNA substitution model was hypothesized as GTR+I+G (lset nst=6; rates= invgamma); 10,000,000 MCMC generations (Yang and Rannala 1997); 4 chains; trees sampled every 1000 runs; 25% of burnin. EF1a: 11 taxa; 598 base pairs (characters); all else remained the same as in the previous analysis. Concatenated COI and EF1a: 11 taxa; 1255 base pairs (characters); all else remained the same as in the previous analyses.

Slide Preparation: Slide preparations were made of the labrum and labiomaxillary complex for both species; the mandibles of the larvae of this genus are too large for slide preparation and were thus excluded from the procedure. The mouthparts were dissected from the head capsule using micro scissors. After the mouthparts were separated from the head capsule the mandibles were gently popped open by applying constant light lateral pressure using sharp forceps. The mandibles were removed by inserting a pin or the end of the sharp forceps between the hypostome-epistome suture. Taking a clean glass slide, the Forh-Berlese media was placed on the slide in the shape of a cross, using a toothpick. The labrum and maxilla were placed on opposite ends of the cross. A small square cutout of cardstock paper was placed at each of the four corners of the cross to act as a buffer to protect the mouthparts from damage by the cover slip. Once all mouthparts were imbedded in the Forh-Berlese media and the paper squares were in place, a clean and dry cover slip was gently lowered over the media. An additional amount of Forh-Berlese media was placed along the edges of the slide cover allowing it to be drawn under the cover slip filling in gaps (Chamorro et al. 2012). Previously prepared cuticle slides were examined and no new slides of body skin were created for this study. The cuticle slides used were a posterior end of body preparation by W.H. Anderson and a complete body skin preparation by R.T. Cotton.

Imaging: Images were taken with an Olympus PEN5 camera mounted on a Zeiss Discovery v8 stereomicroscope with substage illumination and with a Leica compound microscope using Visionary Digital imaging system and stacked with Cartograph. Individual images were taken at different focal planes and combined to create a single image with Helicon Focus. Live larvae were immersed in boiling water for a few seconds and then preserved in ethanol. These specimens were imaged directly and kept hydrated during the focal plane image capture.

Results

All of the specimens, adult and immature, collected in St. Croix as part of the survey are *S. acupunctatus* except for an

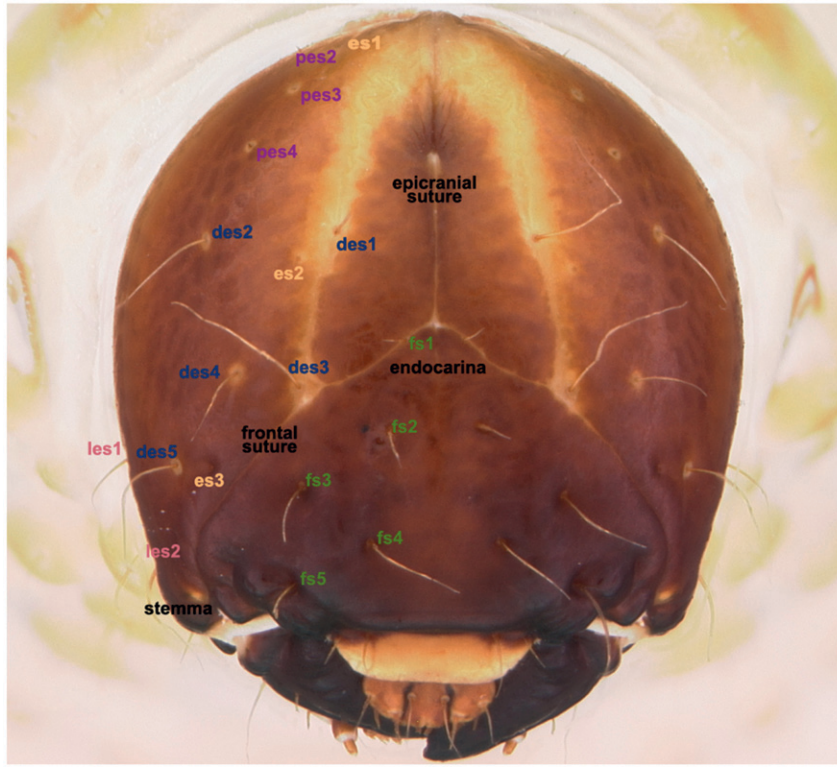

Fig. 4. Head of *Scyphophorus acupunctatus*, anterodorsal view.

adult *Sphenophorus cubensis* (Buchanan, 1936) (Fig. 1J). Both species are here recorded for the first time in St. Croix. *Scyphophorus acupunctatus* is currently present in the north central region of St. Croix (Fig. 1). The survey did not find this species anywhere else on the island (however, see discussion). Species were identified based on morphological characters. The identity of immature *S. acupunctatus* was confirmed using mitochondrial COI gene sequences, or barcode region, and EF1a (Fig. 11). Furthermore, we found multiple generations of *S. acupunctatus* feeding on the same plant.

Phylogenetic analysis of each gene, separately, as well as combined, found considerable phylogeographic structure within *S. acupunctatus* (Fig. 11). Specimens from St. Croix in each case came out as monophyletic distinct from other specimens, which also clustered by location, including Guatemala, Arizona, and most from Mexico (Fig. 11). The sole exception to this pattern was specimen "89" from Mexico; when GenBank COI data from four additional Mexican specimens were included, the four came out clustered in the analysis, while specimen "89" belonged to an entirely different clade. Location and host plant records are provided under material examined. The GenBank specimens and specimen "89" are from different collections.

Scyphophorus acupunctatus was collected from three different host plants; *Agave eggersiana* (Figs 1A, K, L; 2); *Furcraea foetida*, and *A. fourcroydes* (Figs 1E, F). Larvae were collected from the first two plant species. *Sphenophorus*

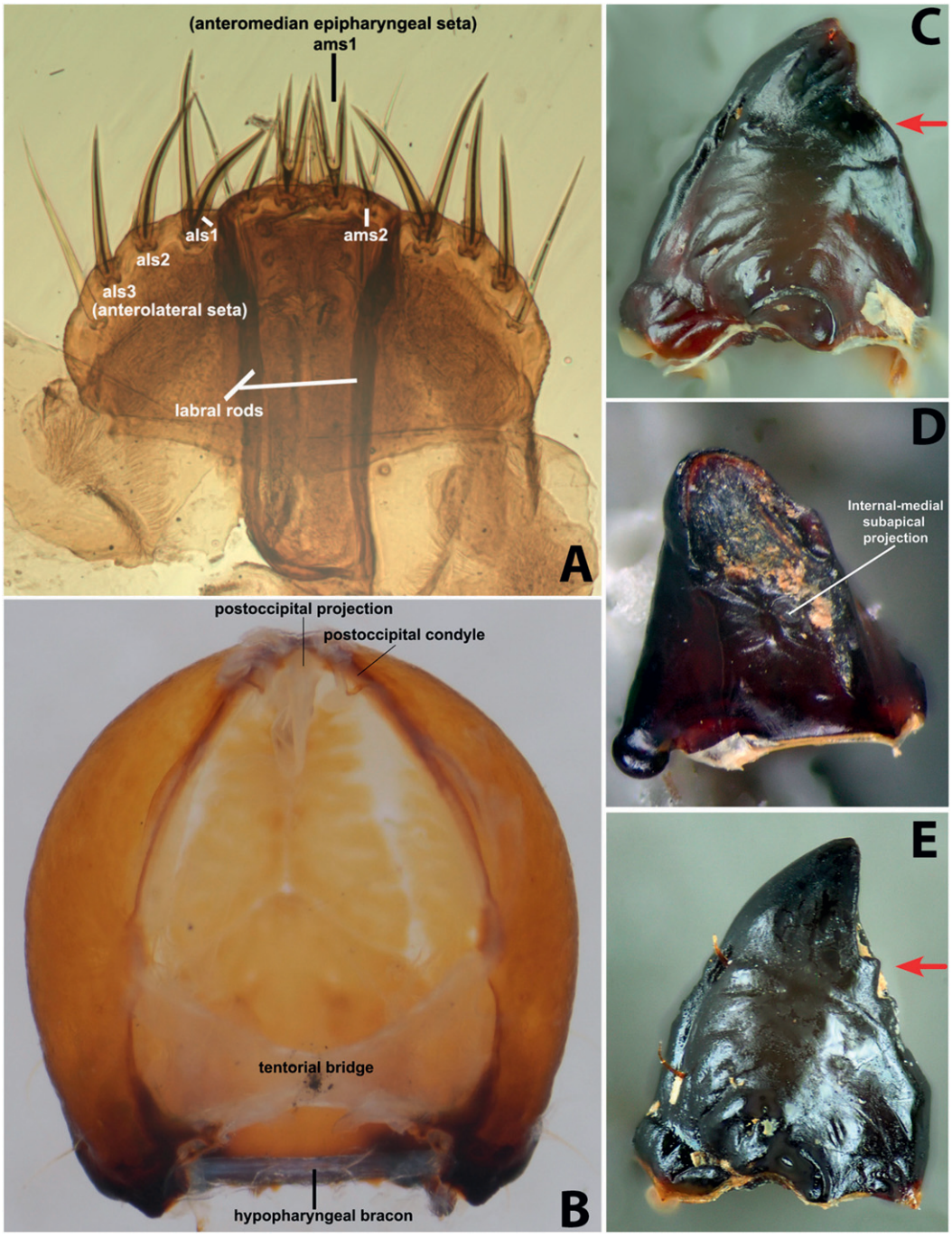

Fig. 5. *Scyphophorus acupunctatus*; A, labrum; B, head, caudal view; C, mandible, dorsal view; D, mandible, mesal view. E, *Scyphophorus yuccae* mandible, dorsal view.

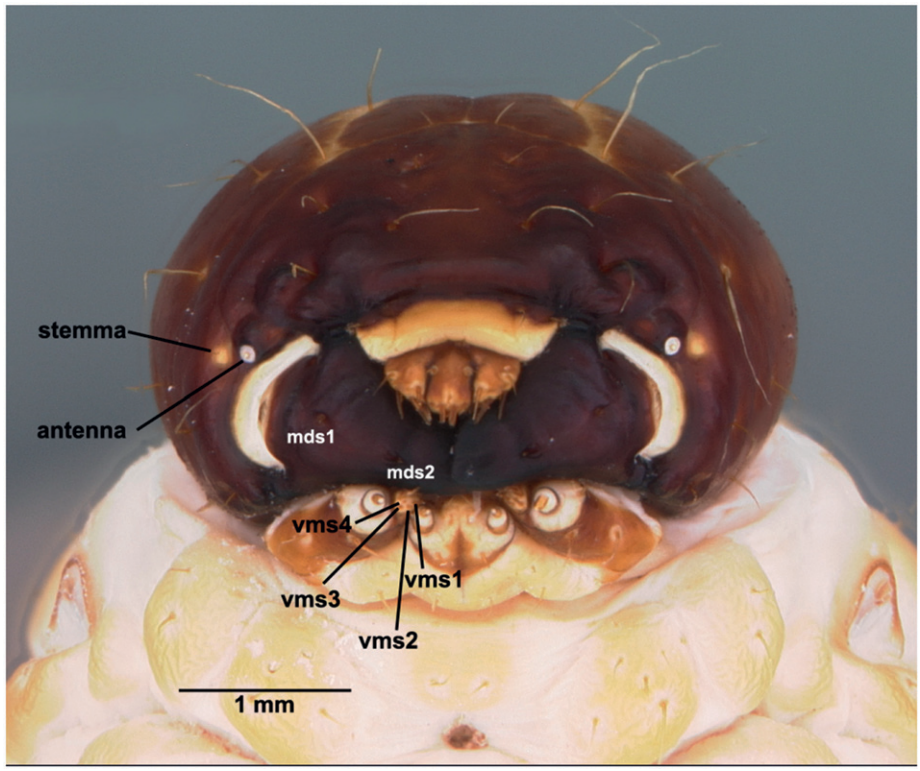

Fig. 6. *Scyphophorus acupunctatus* head, anteroventral view.

cubensis was found on a yet unidentified *Agave* sp. (Figs 1H, I; Fig. 2F). All plant species, except *A. eggersiana* are non-native to St. Croix.

The larva and pupa of *S. acupunctatus* and *S. yuccae* were compared morphologically to identify reliable diagnostic characters. We identified new morphological characters to distinguish the two species; 7 characters of the larvae and 1 of the pupa. These are listed below. Vaurie (1971) identified adult diagnostic characters, but primarily the adults can be distinguished by differences in the antennal club; *S. yuccae* has a visible spongy apex (Fig. 9B), while *S. acupunctatus* does not (Fig. 9D).

We provide an illustrated description of *S. acupunctatus* and diagnoses and keys to distinguish the two species in the genus.

Scyphophorus acupunctatus Gyllenhal

Figures 3–10

Diagnosis.—Among currently known Dryophthorinae larvae, *Scyphophorus* is easily distinguished from other genera by the presence of paired projections on segment IX (Fig. 3B). Adults are recognized by their glabrous integument, the presence ventrally on tarsal article 3 of long, continuous pilosity confined to the apical margin with the remainder of the ventral surface glabrous (Fig. 10D); and the presence of a subtriangular scutellum (widest near the base) among other characters (Vaurie 1971, Anderson 2002, and see key below).

The following are putative diagnostic characters useful for distinguishing larval *Scyphophorus*; 1, labrum: anterolateral epipharyngeal setae (als 1, 2, 3) (Fig. 5A)

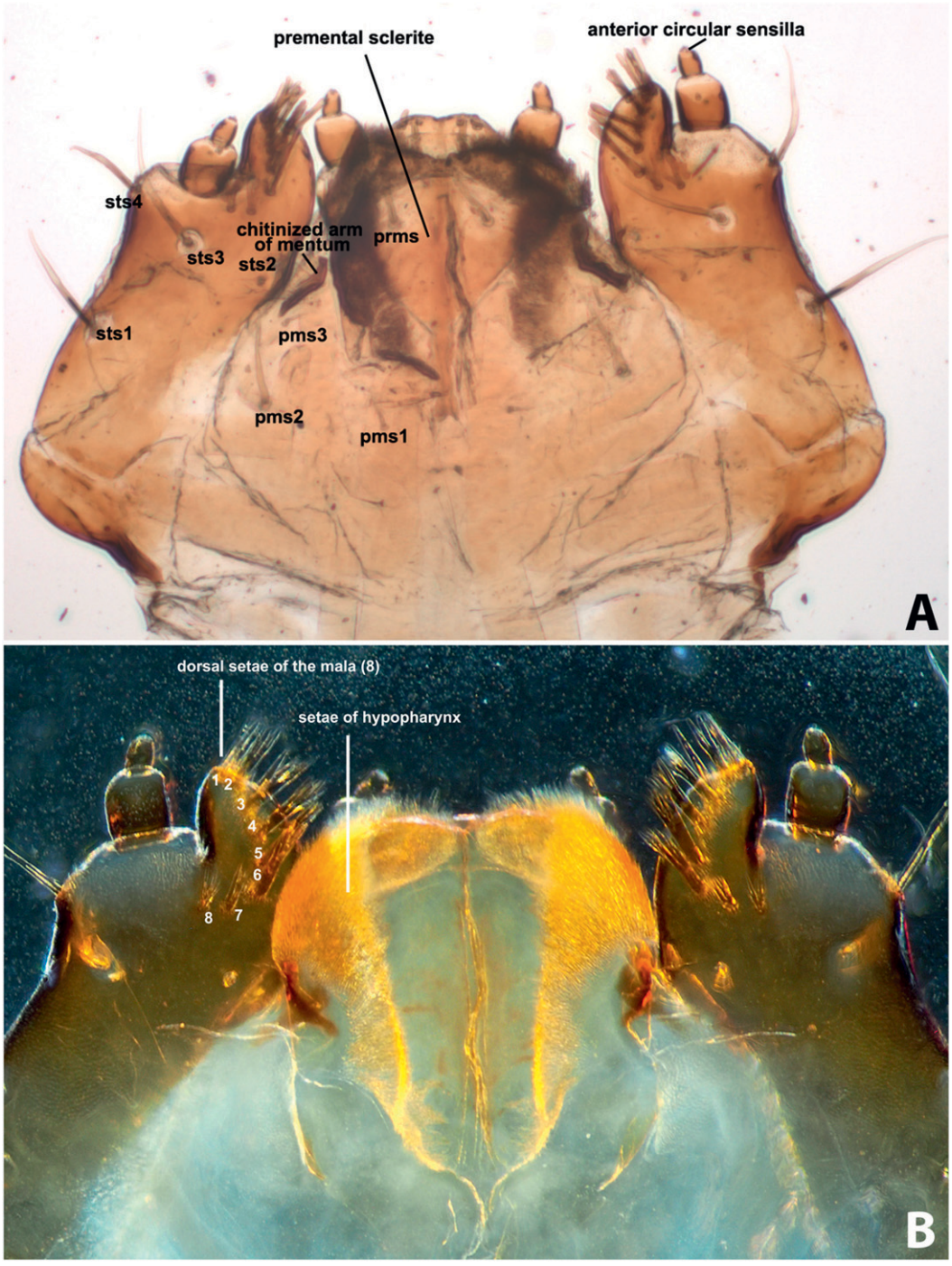

Fig. 7. *Scyphophorus acupunctatus* labio-maxillary complex; A, ventral view; B, dorsal view; anterior circular "spot" =central cluster of peg-like sensilla on second maxillary palpomere.

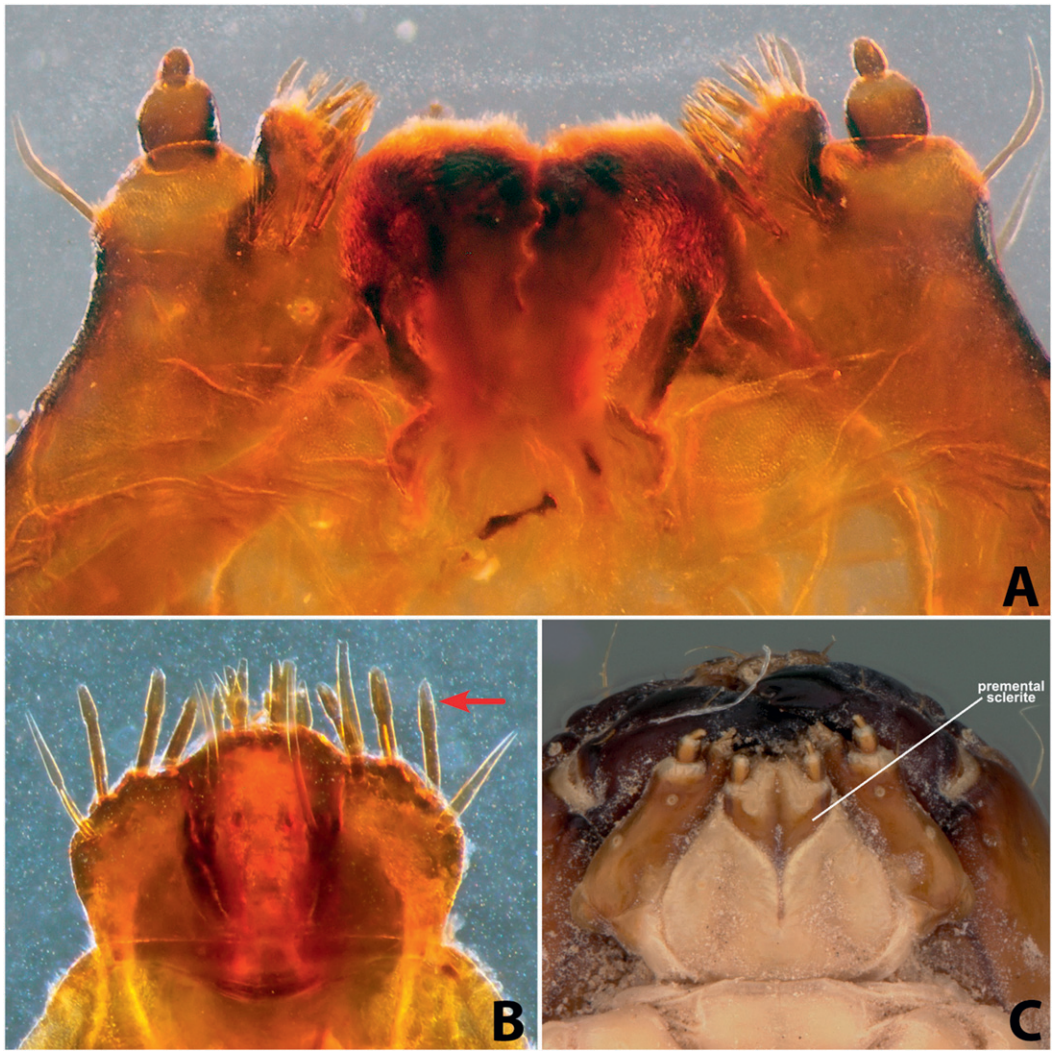

Fig. 8. *Scyphophorus yuccae*; A, labio-maxillary complex, dorsal view; B, labrum; C, labio-maxillary complex, ventral view.

taper to apex in *S. acupunctatus* and are subapically expanded and rounded to the apex in *S. yuccae* (Fig. 8B); 2, anteromedian epipharyngeal seta (ams1) trifurcate in *S. acupunctatus* (at least one, the other may be bifurcate) (Fig. 5A), bifurcate in *S. yuccae* (Fig. 8B); 3, mandibles: the number of marginal (dorsal/ventral internal) subapical projections equal 1 in *S. acupunctatus* (Fig. 5C) and 2 in *S. yuccae* (Fig. 5E); 4, thoracic spiracle (including peritreme): length 2.5 times longer than wide in *S. acupunctatus* (Fig. 3D) and 3-4 times longer than wide in *S. yuccae*; 5, mala: ventral malar seta 2 (vms2) half or less than half the length of vms1 and vms3 in *S. acupunctatus* (Figs. 6, 12) and subequal to vms1 and vms3 in *S. yuccae*; 6, maxillary palpomere: accessory process of second maxillary palpomere prominent in *S. acupunctatus* and reduced in *S. yuccae*; 7, arrangement of peg-like sensillae on second maxillary palpomere located in a central cluster in

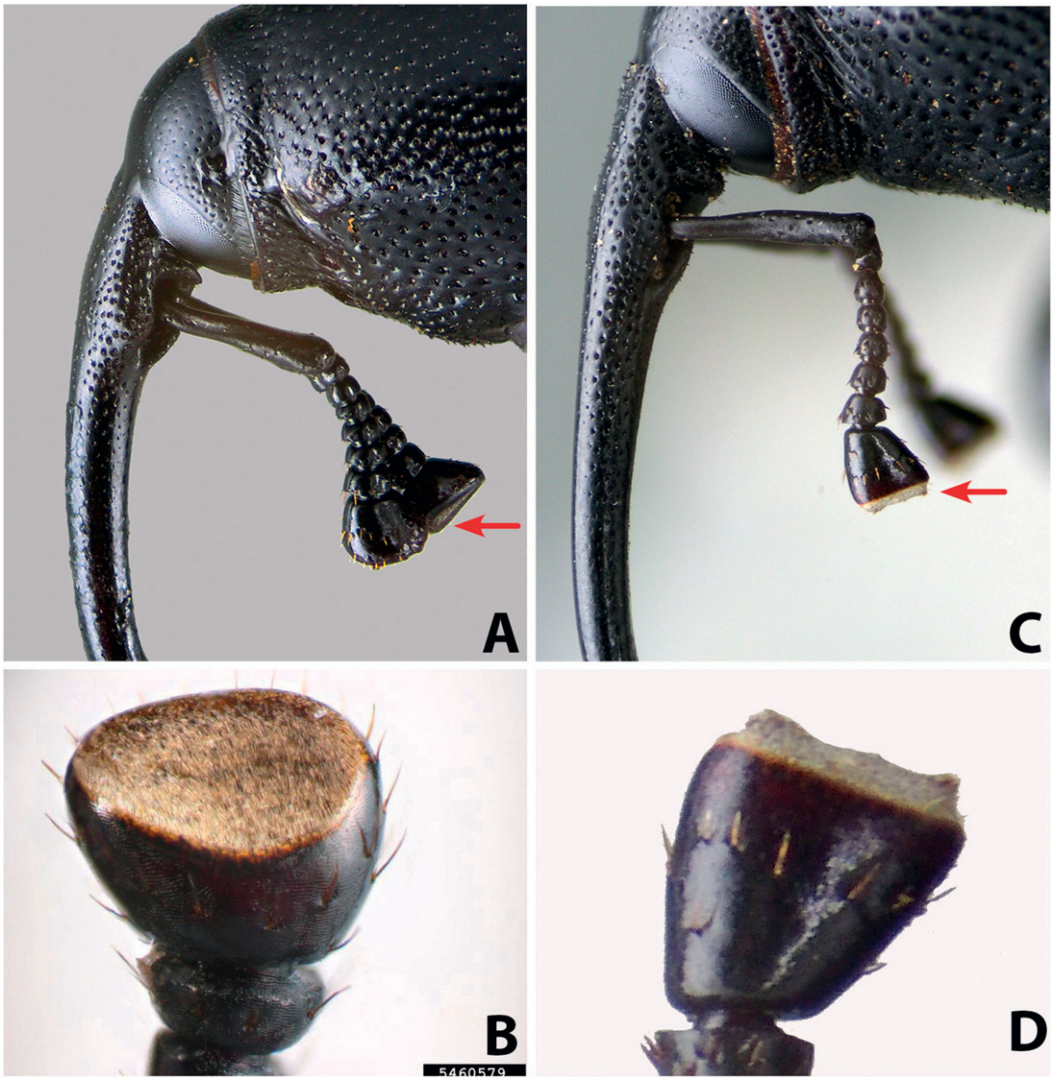

Fig. 9. *Scyphophorus acupunctatus* adult: A, head, lateral view; B, antennal club, detail, caudo-lateral view. *Scyphophorus yuccae*; C, head, lateral view; D, antennal club, lateral view – note the visible spongy apex of the club.

S. acupunctatus (Fig. 7) and on the edge of the apex with ends of the sensillae directed outward in *S. yuccae*.

Characters to distinguish pupae of *Scyphophorus*: number of tubercle-borne setae on rostrum equals 10 in *S. acupunctatus* (Fig. 10A) and 8 in *S. yuccae* (Fig. 10B).

Description of the mature larva of *Scyphophorus acupunctatus*

Body. Size: 12—15 mm. Overall color (affected by method of preservation): creamy white; curvature: C-shaped; overall shape: robust; expansion of abdomen: ventrolaterally expanded from segment III-V then abruptly narrowed; cuticle surface: finely asperate; location of cuticular spicules: throughout.

Head: free; frontal suture: complete, extending to articulating membrane of

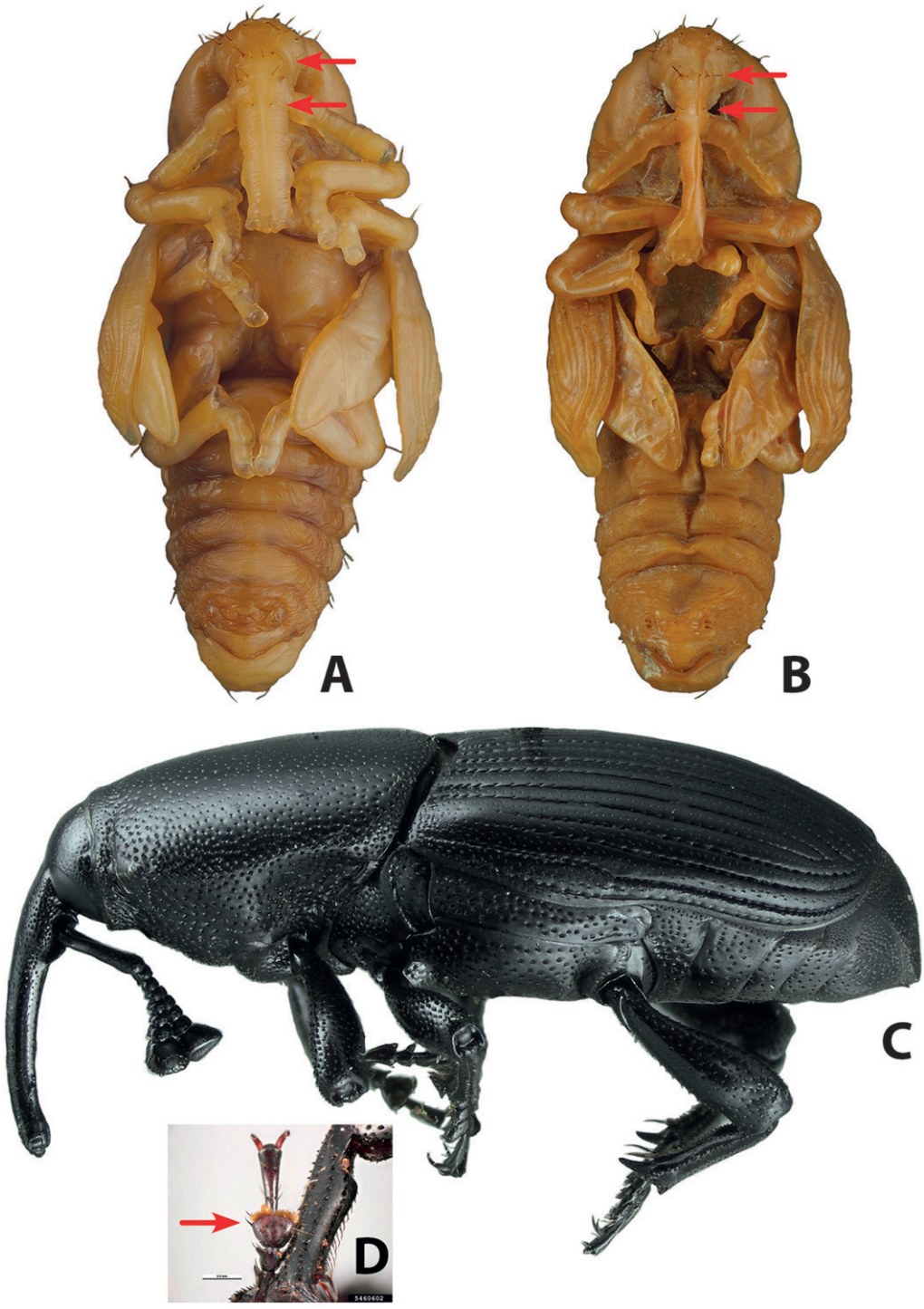

Fig. 10. *Scyphophorus* spp. pupae; A, *S. acupunctatus*, ventral view; B, *S. yuccae*, ventral view. *Scyphophorus acupunctatus* adult; C, lateral view; D, detail of tarsomeres showing genus level character of characteristic setal pad arrangement.

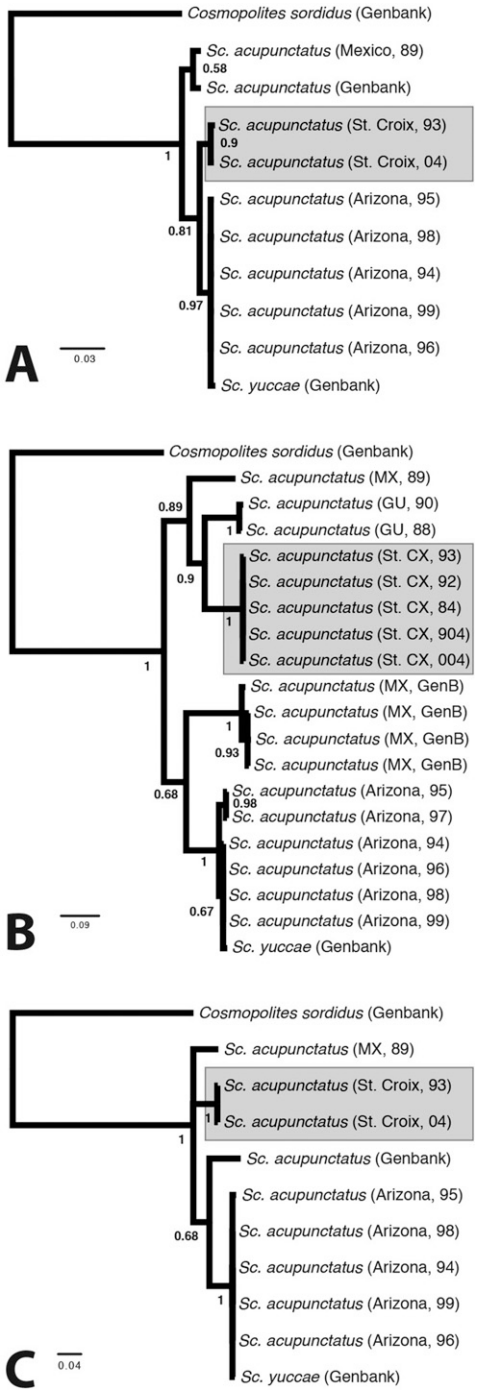

Fig. 11. Bayesian inference 50% majority rule cladogram; A, EF1a gene; B, COI; C, concatenated analysis of COI and EF1a genes. Values at the internodes are Bayesian posterior probabilities.

mandibles; frontoclypeal suture: distinct; convergent, non-pigmented stripes: present. Anterior stemmata: present; posterior stemmata: absent. Antenna: with a membranous, cushion-like basal segment and an apical, convex sensorium. Number of setae on frons (fs): 10 (5 pairs). Hypopharyngeal bracon: without sclerome; epicranium postocciput: with hyaline posterior, acute inflexion; Postoccipital lamina (projection): apparently present with apodemes. Postoccipital condyles (cervical plates): present.

Mouthparts: Fine setation on either side of clypeus-epistomal suture: absent. Number of anterolateral epipharyngeal setae (als): 3 on each side; anterolateral epipharyngeal setae (als1, 2, 3): tapering to apex; anteromedian epipharyngeal seta (ams1): trifurcate (at least one). Mandibles: without mola; shape of mandibles: deltoid; mandible symmetry: symmetrical; size of mandibles: approximately 1/3 length of head; internal-medial subapical projection on mandible: present; number of marginal (dorsal/ventral; internal) subapical projections on mandible: 1; incisor cusps of mandible: absent. Setation of lateral lobes of hypopharynx: setose; placement of setae on hypopharynx (labium): laterally, tapering proximally, broadening distally shrouding apex towards venter; chitinized arm of mentum (postlabial strut or postlabial bracon) elongate (4 times longer than wide). Number of dorsal setae of the mala: 8; mala dorsally: with branched seta; ventral branching of dorsal malar setae (progression): increased branching proximally; asperites among setae of dorsal surface of mala: present; ventral malar setae: unbranched; ventral malar setae 2 (vms2) relative length: half or less than half the length of vms1 and vms3; ventral malar seta 4 (vms4): greatly reduced (almost peg-like sensillum) (Fig. 12); vms5 apparently absent (Fig. 12). Lacinial lobe or spine: absent. Setae on

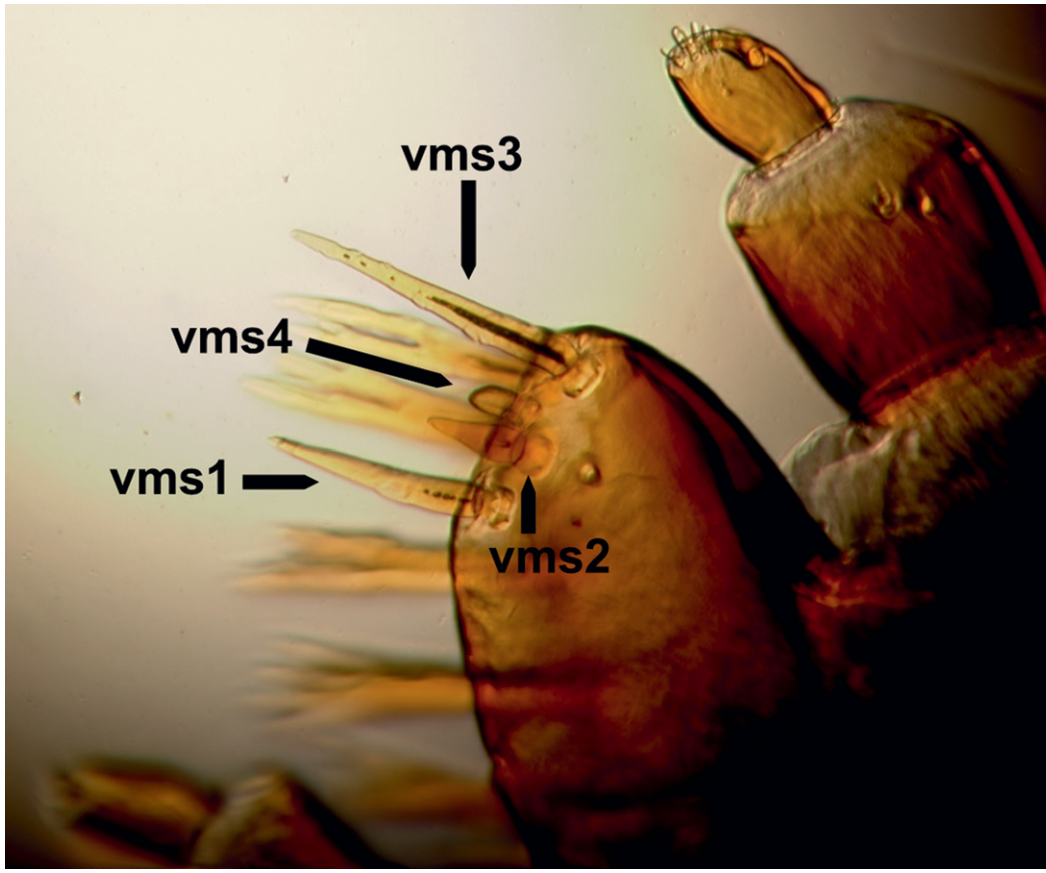

Fig. 12. *Scyphophorus acupunctatus* larva, left mala (detail), ventral view.

stipes (sts1, 2, 3, 4): broadening medially and narrowing to apex. Maxillary articulatory lobes: absent; maxillary palp: 2-segmented; accessory process of second maxillary palpomere: prominent; arrangement of peg-like sensilla on second maxillary palpomere: pegs located in central cluster; apical sensory peg-like sensilla of second maxillary palpomere: circular. Position of premental setae: medially, between forks of premental sclerite; pigmentation of premental sclerite: sclerotized. Position of postmental setae: pms3 and pms2 lateral and pms1 medial, distance among pms subqual to each other.

Thorax. Legs: absent; pedal area: distinct (bulging and setose). Thoracic terga: without patches of spicules (asperities). Thoracic spiracle: on prothorax; shape of thoracic spiracles: straight; length of thoracic spiracle (including peritreme): 2.5 times longer than wide. Setae on pronotum: 7 setae on each side; prodorsum of mesonotum: 1 pair of setae (1 on each side); seta of meso- and metathoracic epipleurites: not equal in length (shorter on metathorax); prodorsum of the metanotum: 1 pair of setae. Alar area of the mesonotum: 2 pairs of setae (2 on each side); alar area of the metanotum: 2 pairs of setae (2 on each side). Space between eusternum and sternellum of prosternum with: small circular opening.

Abdomen. Segment VIII and IX: flattened dorsally. Setae shape: short and fine (I-VII); long and coarse (VIII-IX).

Abdominal segment folds (I-VII): 3. Setae between intersegmental fold (dorsal fold) and scutum: absent. Abdominal pleura: subdivided into 2 or more superimposed lobes. Spiracular air tubes: well developed; spiracles of segments I-VII: prominent; spiracular airtubes of abdominal segments I-VII: directed dorsally. Posterior margin of abdominal segment IX: with a pair of projections; shape of posterior abdominal projections: 2x longer than wide (long and broad; reference widest section); setation of posterior abdominal projections or sides each with: 3 elongate setae. Shape of preanal lobe: posteromedially triangular, expanding anterolaterally; setae of preanal lobe: 2 anteriorly. Shape of median lobe: subrectangular; number of setae on median lobe of the anus: 2. Lateral anal lobes: present; shape of lateral lobes: subtriangular with medial, weakly sclerotized expansion; number of setae on lateral lobes: 1 on sclerotized expansion and 1-2 apically.

Larval behavior. Prepupation behavior on host plant: Burrows towards roots to pupate (Waring 1987; Huxman et al. 2007).

Description of the pupa

Epicranial setae: present. Number of tubercle-borne setae on rostrum: 10. Inner body: yellowish; theca: transparent. Mandibular theca: lacking setae. Setae on pronotum: present; distance between setae on pronotum: distance subequal. Setae of mesonotum: present. Femoral apex: with 1 to 2 setae. Last pair of legs: largely exposed, not covered by pterothecae.

Material Examined.—*Scyphophorus acupunctatus:* BRITISH VIRGIN ISLANDS: Guana I, 27-28-x-/ ’01 B&B Valentine (WIBF). DOMINICAN REPUBLIC: Prov. Pedernales; ca. 35km NNW Cabo Rojo, 1430m, El Aceitillar; 09SEP1988, pine forest; M. Ivie, Philips & Johnson (WIBF). GUATEMALA: Port Miami Plant Inspection Station (USDA APHIS PPQ interception), 15.xi.2013 (USNMENT01070988, adult); 12.ix.2014 (USNMENT01070990, adult). HAITI: Kenscoff, 18°27’N, 72°17’W; 10/5/74 (WIBF). MEXICO: Sonora, Magdalena (USNMENT01075012, larva); Morelos, Cuernavaca, 16.xii.1938 (larval slide preparations); Port Pharr US Customs and Border Patrol (USDA APHIS PPQ interception) 8.ix.2014 (USNMENT01070990, adult); 25.v.2014 (USNMENT01070989, adult). PUERTO RICO: Guánica State Forest, 7.iii.2014, coll. Torres-Santana and Hamilton (MEBT, adult) ex *A. sisalana*; USA: Arizona: Fort Grant, 16.vii.1897 (USNMENT01075007, adult and larvae; USNMENT01075009, larva); San Pedro River, date: N/A (USNMENT01075010, larva); Cochise, S.W. Research Station, 5 mi W Portal, 5400ft 11.vi.1956 (USNMENT01075011, larvae, pupae and adults); Coronado National Forest Road to Rustler Park, on *Agave* 31.905730, -109.278572, 09.viii.2014, coll. M. L. Chamorro, ex. *Agave palmeri* Engelm. (USNMENT01070995, USNMENT01070996, USNMENT01070997, USNMENT01070998, USNMENT01070999, adults); Cave Creek Trail, 10.viii.2014, Coll. M. L. Chamorro, ex: *Agave palmeri* (USNMENT01070994); [Santa Cruz], Nogales, N/A (USNMENT01075005, larva); N/A (USNMENT01075013, larval slide preparation); N/A (USNMENT00977002, larvae). U.S. VIRGIN ISLANDS: St. Croix, Christiansted, driveway to Salt River Bay National Historical Park and Ecological Preserve 17.79, -64.76, 35ft, 19.xii. 2013, ex. *Furcraea foetida*, coll. Cherubin, Torres and Díaz (USNMENT01070984, larva); (USNMENT01070992, adult ex: *Agave eggersiana*); (USNMENT01070993, adult ex: *A. fourcroydes*); Est. Mary’s Fancy, [Coordinates omitted because this is a private residence], 25.xi.2014, 280m, leg. J. Keularts ex: *A. eggersiana*, base (USNMENT01075004, adult;

USNMENT01074909, adult; USNMENT 01074904, adult; USNMENT01173574, 2 larvae); St Thomas, Est. Botany Bay, 28.vi1994, C. Robles, in century plant (WIBF).

Scyphophorus yuccae: USA: California, Arrowhead, April 1892 (USNMENT01075006, larva), USA: n/a: (USNMENT01075001, pupa; USNMENT01075000, pupa; USNMENT01075008, larva, pupae); Arizona, N/A (larval slide preparations). PANAMA: Canal Zone: Barro Colorado Island, 17-21.ii.1963. Blake & Cochran, colls. ex. *Hesperoyucca whipplei* (=*Yucca whipplei* Torr.) (1428 adults).

GenBank:

Cosmopolites sordidus EF1a: AY131140.1; COI: AY131111.1

Scyphophorus yuccae COI: AY131110.1; EF1a: AY131139

Scyphophorus_acupunctatus_MX_ GenBank COI: AY131122;

Scyphophorus_acupunctatus_MX_ GenBank COI: JX134900;

Scyphophorus_acupunctatus_MX_ GenBank COI: JX134902;

Scyphophorus_acupunctatus_MX_ GenBank COI: JX134907

Scyphophorus_acupunctatus_EF1a: AY131151

Comments.—Distinguishing characters are provided based on current observations. Our sample-size of *Scyphophorus yuccae* larvae was limited and it may be possible that some of the chaetotaxy and mandibular characters are more variable than here indicated. Furthermore, mandibular characters may be plastic and affected by usage, however this may only be determined with a much larger sample size. Finally, different larval instars may exhibit slight differences in morphology. This was not studied.

Key to larvae of *Scyphophorus* (based on a limited sample of *S. yuccae*)

1. Anterolateral epipharyngeal setae (als 1, 2, 3) taper to apex (Fig. 5A); anteromedian epipharyngeal seta (ams1) with at least one trifurcate (Fig. 5A); mandibles each with one marginal subapical projection (Fig. 5C); thoracic spiracles (including peritreme) 2.5 times longer than wide (Fig. 3D); ventral malar seta 2 (vms2) half or less than half the length of vms 1 and 3 (Fig. 6); accessory process of second maxillary palpomere prominent; peg- like sensillae on secondary maxillary palpomere located in a central cluster (Fig. 7) . . . . . .*S. acupunctatus*

– Anterolateral epipharyngeal setae (als 1, 2, 3) subapically expanded and rounded to apex (Fig. 8B); anteromedian epipharyngeal seta (ams1) bifurcate (Fig. 8B); mandibles each with two marginal subapical projections (Fig. 5E); thoracic spiracles (including peritreme) 3-4 times longer than wide (Fig. 3D); ventral malar seta 2 (vms2) subequal to vms 1 and 3; accessory process of second maxillary palpomere reduced; peg-like sensillae on secondary maxillary palpomere located on the edge of the apex with ends of sensillae directed outward ..... *S. yuccae*

Key to pupae of *Scyphophorus*

1. Ten tubercle-borne setae on rostrum..... ..... *S. acupunctatus*

– Eight tubercle-borne setae on rostrum.... ..... *S. yuccae*

Key to adults of *Scyphophorus* (from Vaurie 1971)

1. Antennal club with spongy apex retracted, concave, not visible in lateral view (Fig. 9A, B); antennae with segment 2 of funicle of same length as 3 (Fig. 9A); terminal segment twice as wide as long (Fig. 9A); scutellum smaller, scarely wider than base of sutural interval (Fig. 9A); elytra with interval very finely, shallowly punctate; apices truncate .....*S. acupunctatus*

– Antennal club with spongy apex truncate, somewhat carinate, visible in lateral view as narrow line (Fig. 9C, D); antennae with segment 2 of funicle longer than 3 (Fig. 9C);

terminal segment less transverse (Fig. 9C, D); scutellum larger, longer, twice as wide as base of sutural interval; elytra with intervals deeply punctate in single line; apices obliquely retracted to suture ..... *S. yuccae*

DISCUSSION

The collaborative, multiagency survey of St. Croix discovered the presence of *S. acupunctatus* in North Central St. Croix, however it was not found in any of the *A. eggersiana* critical habitat sites (Figs 1, 2A). Following the survey one of us, JK, found this species on previously healthy, non-flowering *A. eggersiana* plants on his property in Central St. Croix (Figs 1, 2B, C, D). This record may signify a slow, but steady movement of the weevil towards suitable host-plant sites across the island and may eventually reach these critical habitats where plants of *A. eggersia*na are currently present (USFWS 2014a).

We therefore strongly recommend monitoring for the presence of *S. acupunctatus* on the eastern half of the island where the *A. eggersiana* naturally occurs. A combination of pheromone lures (Rodríguez Rebollar 2011, Rodríguez-Rebollar et al. 2012) and visual examination of agave rosettes is strongly recommended in order to detect, monitor, and control the spread of this weevil. While treacherous, due to the presence of sharp, stiff apical spines on the plant (Fig. 2C), it is important to carefully inspect between the leaves where the adults are commonly found. Other signs to look for are the presence of senescing (reddish color on yellowing leaves) or collapsed agave plants (Fig. 2D); decomposition and fermentation at the base of the agave (Fig. 2E), and the presence of characteristic oval-shaped feeding holes (Figs 2E, F). It is important to keep in mind that the larvae are endophytic and not easily detected externally. In addition, we recommend removing the infested plants however, this will require permits prior since it is an endangered species. As a rapid response measure, were the invasive weevils arrive to an intact and stable population of *A. eggersaiana*, we suggest the following resource that offers management techniques: http://www.eastmark.com/wp-content/uploads/2015/04/landscape_year-round_agave_snout_weevil.pdf.

The possibility exists that the presence of the weevil may be a result of stressed plants whereby the weevil is cueing into stress volatiles and the weevil may not be the primary culprit for the plant's decline. Perhaps the affected plants on the north central and central parts of St. Croix were experiencing higher than normal levels of stress making them vulnerable to attack by the weevil pest, while plants found in more suitable habitats along coastal cliffs on the eastern part of the island are not. Nonetheless, monitoring is crucial given the endangered status of *A. eggersiana*, the current planetary climactic changes that may lead to unexpected stresses, and the now confirmed presence of an oligophagous weevil pest with an ability to disperse, colonize, and destroy host plants. In addition, *A. eggersiana* may lack natural defenses against the weevil, unlike *A. palmeri* (Waring and Smith 1986), which co-evolved with this weevil, and is therefore vulnerable to its attack. The presence of microbial endosymbionts in the weevil may further promote the decline and subsequent death of the plant (Waring and Smith 1986).

Scyphophorus acupunctatus may have arrived on the island through a number of ways: 1, by dispersing from neighboring Puerto Rico or another nearby island where it previously became established; or 2, by traveling with a non-native agave plant or cargo.

The molecular data analyzed in this study confirms the identity of the immature forms and serves to bridge the more taxonomically

important adult with the usually character poor immatures. This study provides an authoritatively identified DNA library for *S. acupunctatus* that can be relied upon for future studies or identifications.

Phylogenetic analysis of both genes separately and together indicates a clear pattern of substantial phylogeographic structure with specimens clustering by geographic location (Fig. 11). Although our sampling is limited, this pattern suggests the presence of cryptic species or allopatrically diverged populations (Fig. 11). Furthermore, that all specimens from St. Croix shared the same COI sequence as well as the same EF-1a sequence, distinct from others in the study, indicates that we have yet to sample the source population for S. *acupunctatus* in St. Croix. Further investigation of phylogeographic as well as host structuring in *S. acupunctatus* would make an important contribution towards both understanding whether cryptic species are present and determining the source population(s) for introduced populations of *S. acupunctatus.* If molecular evidence for cryptic species is found, careful morphological study needs to be performed to uncover diagnostic characters for each group in order to make it possible to identify at least adult specimens using morphology. *Scyphophorus acupunctatus* currently has 4 junior synonyms: *S. interstitialis* Gyllenhal, 1838; *S. anthracinus* Gyllenhal, 1838; *S. asperulus* (LeConte, 1857); and *S. robustior* Horn, 1873 (Vaurie 1971; O'Brien and Wibmer 1982) placed there by LeConte (1876). The validity of these names may have to be considered as more data becomes available. According to our results, we suspect that the sequence of *S. yuccae* obtained from GenBank actually corresponds to a misidentified specimen of *S. acupunctatus* (Fig. 11).

During this study we attempted to compile published host-plant associations to determine whether host-plant preference can be used to distinguish *S. acupunctatus* from *S. yuccae* in the field. However, some published records are suspect due to misidentifications. Maya et al. (2011) reported a new host association for *S. acupunctatus, Pachycereus pringlei* (S. Watson), but the weevil imaged in the publication appears to be another Dryophthorinae, *Cactophagus spinolae* (Gyllenhal). Current evidence suggests *S. yuccae* to have a tight host-plant association with only a few members of the subfamily Agavoideae, namely *Hesperoyucca* (Engelm.) Baker and *Yucca* L. However, given the apparently broad host-plant range of *S. acupunctatus* and reports of its occurrence even on *Yucca* (Pott 1975), the fidelity of the weevils to host-plant is likely unreliable as a diagnostic tool.

The paired structures labeled as 'chitinized arm of mentum' in Figs 7A, B may be a putatively informative character when inferring Curculionoidea relationships, as similar structures (may or may not be homologous) occur in early divergent curculionoids and chrysomeloids (Marvali 2005: character 21 state 1; Marvaldi pers. comm.). This structure has not been previously recorded in Curculionidae (Marvaldi pers. comm.).

Careful scrutiny was placed on the setation of the ventral mala, which was cleared, slide mounted, imaged (Fig. 12) and re-examine under high magnification. The setal configuration of the ventral mala apparently lacks a pair of minute, contiguous, ventral malar setae at the site of vms4 in Fig. 12 [unlike Marvaldi 1997; Fig. 26A for *Cosmopolites sordidus* (Germar, 1824); Marvaldi pers. comm.]. This configuration is unexpected, but based on our examination; only 4 clearly visible, socketed setae are present.

This is the first record of *S. acupunctatus* in St. Croix, where it represents a threat to an endangered century plant. The necessary morphological, behavioral and molecular tools to unequivocally identify the weevil, or portion thereof, at any stage are provided. Detection and rapid identification of this weevil is crucial towards its control and to mitigate the risk it poses to valuable and vulnerable natural resources.

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