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CLARIFICATION OF THE STATUS OF *CHIRONOMUS JAVANUS* KIEFFER, 1924 AND *C. VITELLINUS* FREEMAN, 1961

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Abstract

The status of the closely related species *Chironomus javanus* Kieffer, 1924 and *C. vitellinus* Freeman, 1961 has been largely confused and *C. vitellinus* even considered to be a junior synonym of a very widely distributed *C. javanus.* However, a comparison of the available mitochondrial COI barcode sequences reveals that there are two groups of sequences with consistent differences between them. Further, the geographic origins of these sequences indicates that *C. vitellinus* is much more widely distributed than *C. javanus.* This interpretation is confirmed from the details of various descriptions, with differences particularly in the male hypopygium and the larval premandible. *Chironomus vitellinus* is also found in the New World in Florida and Puerto Rico, indicating that the species is probably distributed through most of the tropical region. However, *C.* sp. "Florida" of Epler (2001), known only as a larva, and with a multi-toothed premandible has been determined to be a separate species.

Introduction

Current literature on *Chironomus javanus* suggests that it is a widespread species in tropical regions from Central Africa to the islands of Micronesia in the Pacific. On the other hand *C. vitellinus* is either not considered or regarded as a synonym of *C. javanus* (Chaudhuri et al. 1992, Martin 2022). A reassessment of the literature, along with additional specimens and, particularly, a comparison of the available DNA barcode sequences as advocated by Desalle et al. (2005), reveals that this picture is highly flawed. Rather, as will be outlined below, *C. javanus* and *C. vitellinus* are closely related species with relatively different distributions that apparently only overlap in a small area of India and the Malaysian Peninsula. It is not the purpose of this paper to re-describe these species but rather to provide the evidence relevant to confirming that they are distinct species and to correct the data on their respective distributions. The confusion seems to have arisen accidentally from Tokunaga (1964) who described the Micronesian populations of *C. vitellinus* as *C. javanus.* Even if Tokunaga was aware of Freeman's description of *C. vitellinus* just 2 years previously, he very likely would not have recognized that it was a different species as Freeman did not illustrate the tergite IX setae, which are the most easily recognizable character for distinguishing the two species. Subsequently, any specimens with the arrangement of TIX setae figured by Tokunaga were identified as *C. javanus.* It will be shown that this is not correct as the setation in the two species is quite different.

When these factors are taken into consideration, it becomes clear that most descriptions attributed to *C. javanus* in fact refer to *C. vitellinus* which is very broadly distributed, with individuals reported from Malawi, Africa to Japan, the Melanesian Islands of the Pacific Ocean and, as reported here, to Florida, U.S.A. and Puerto Rico. *C. javanus,* rather than being very broadly distributed, appears to have a restricted distribution in India and Peninsular Malaysia. The morphological, cytological and mitochondrial COI characters that separate these two species are outlined below.

Material and Methods

Material examined has included larvae, pupae, and adults from various localities including:

Chironomus javanus from Bukit Merah Agricultural Experimental Station (BMAES) Permatang Pauh, (Dr. S.A. Al-Sharmi) and Mendang, Penang (Warrin Ebau), with other information drawn from published works (Kieffer 1924, Johannsen 1932, and Chaudhuri et al. 1992).

Chironomus vitellinus from Mareeba, Sarina and Townsville in Queensland, Australia; a paratype male from Mafulu (Natural History Museum, London) and larvae from Sogeri, Central Province, Papua New Guinea (N.V. Dobrotworsky); Bukit Merah Agricultural Experimental Station (BMAES) Permatang Pauh, Penang (Dr. S.A. Al-Sharmi); Labasa, Vanua Levu and Nadi, Viti Levu, Fiji; Laboratory colony from Shizuoka, Shizuoka Prefecture, Honshu, Japan (Prof. H. Hashimoto); Blantyre, Malawi, Central Africa (Dr. A.J. McLachlan); Alachua, Charlotte and Wakulla Counties, Florida, U.S.A., and Puerto Rico (J.H. Epler); with other information from numerous published works, mostly as *C. javanus* (Freeman 1961, Tokunaga 1964, Hashimoto et al. 1981, Sasa and Hasegawa 1983, Hashimoto 1984, Cranston 2007, Al-Sharmi et al. 2012, Pramual et al. 2016).

Morphological terminology follows Sæther (1980), Webb and Scholl (1985) and Vallenduuk and Moller Pillot (1997). Abbreviations include JM – Jon Martin; JHE – John H. Epler; NA - North America.

Chromosome preparation techniques and the identification of chromosome arms are as previously described (e.g. Martin et al. 2006).

MtCOI sequence was obtained from ethanol/acetic acid fixed specimens (mostly larvae) for the conventional mitochondrial cytochrome c oxidase subunit I (COI) fragment using the Folmer et al. (1994) primers: LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). In some cases the condition of the specimens was such that the barcode region had to be amplified in two sections, using the reverse primer COI-Na-2 (5’- AGATAAAGGKGGATAAACWGTTCA-3’) for the 5’ section (Martin, 2023), and the forward primer of Carew et al. (2013) (5’-CCHCGAATAAATAATATAAGWTTYTG-3’) for the 3’ section, with PCR products sent to Macrogen Inc. Seoul, Republic of Korea for sequencing.

The resulting sequences were compared to sequences identified as *C. javanus* in the Barcode of Life Data Systems (BOLD) database (BIOUG21394-A08, BIOUG26140-B05, GMBCA5882-15 (all Bangladesh); ATISA8024-16, BIOUG-G04, MYP3811, MYA, MYA2 and MYA3, P_W02_132, P_W06_145 (all Malaysia), DIQTB182-10 (Australia), GMBCA5882-15, Mr-23 (China) and in GenBank (DQ648203 (Japan); KF408077 (India); KP462106 (Singapore); KT212984, KT212989, KM013379, KM013380, KM013385 (all Thailand); JF412082, JF412083, JF412084 and JF412085 (all Korea), JN298748 (Australia); MW201293 (China) along with sequences that have been lodged in GenBank as *C. vitellinus*: (OR367025 and OR486048 (both Malaysia); OR367026 (Papua New Guinea).

Results

An examination of the published figures of the male hypopygium of supposed *C. javanus* specimens subsequent to that of Johannsen (1932) shows that only those of the Indian specimens of Chaudhuri et al. (1992) are equivalent with a narrow tubular anal point and no patch of setae on tergite IX (TIX). All others are identical to that of the *C. javanus* of Tokunaga (1964) in having a small expansion at the end of the anal point (as in Freeman’s original figure) and a patch of about 5-17 setae in individual pale patches on TIX which, although not shown in Freeman’s original figure, were observed on the paratype male from Mafulu, Papua New Guinea (Fig. 1). This was the first clue that *C. vitellinus* was actually quite widely distributed.

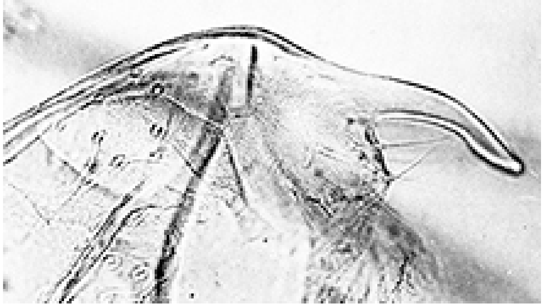

Figure 1. Terminal abdomen of paratype male from Papua New Guinea showing the TIX setae (at left) and narrow anal point.

There are also identification features for *C. javanus* in the larva: the central teeth of the mentum do not arise below the level of the other teeth as they do in *C. vitellinus* (Fig. 6) and the premandible which has multiple teeth and for which Peter Cranston (pers. comm.) determined that, if examined closely, there are 6 teeth in *C. javanus* and 7 teeth in *C. vitellinus.* Cranston (2007) illustrates a *C. vitellinus* premandible which clearly has 7 teeth but is labelled as *C. javanus* because at that stage he had not recognised the difference between the two species. Hashimoto (1984) claimed to have studied *C. javanus* but his figures indicate he actually had *C. vitellinus.* He states that the premandible has 6 teeth but does not provide a figure to confirm this count since the 7th tooth is small and easy to overlook if the premandible does not lie in an optimal orientation. However, the Central American samples available to us also have only 6 teeth, suggesting that there may be polymorphism for tooth number in *C. vitellinus.* One possibility is that the U.S. and Puerto Rico specimens actually arose by human transport from Japan – a situation recognised previously for a number of species in South America and southern U.S. (e.g. *C. striatipennis* Kieffer which is suggested to have originated in China or perhaps Japan – see Amora et al. 2015).

Dr. Al-Sharmi provided larvae and adults from his samples collected in rice paddies in Pulau Pinang, Malaysia. These samples contained both *C. vitellinus* (most common and the species described by Al-Sharmi et al., 2012) and *C. javanus.* It also included a small number of slide mounted adult males, most of which were *C. vitellinus,* but one rather distorted specimen fitted the description of *C. javanus* and agreed with the characters of adult males from previous descriptions except of Johannsen (1932) and Chaudhuri et al. (1992 in that there were two rows of long setae, an anterior one of 7 setae and a more posterior row of 6 setae (Fig. 2) on TIX. This specimen had simply been cleared and then mounted whole on a slide. This suggests that these setae may commonly be lost in preparation of specimens for slide mounting.

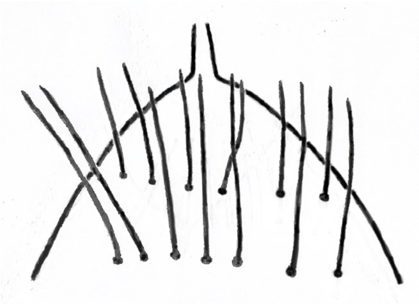

Figure 2. TIX of *Chironomus javanus* showing the long setae.

Barcode sequence was obtained from some larvae of this sample and produced some very informative results (discussed below). Consequently an analysis was made of the available sequence for *C. javanus* in the BOLD, where all samples were in the same Bin (BOLD:AAG6924) as our samples of *C. vitellinus,* or GenBank databases. The sequences were aligned so that the actual base sequence could be analysed as recommended by DeSalle et al. (2005). The samples could be separated into two groups which differed at 47 sites, 3 of which were polymorphic in *C. vitellinus* (Fig. 3)*,* and mostly in the 5' region. The largest group (23 specimens) included my *C. vitellinus* samples, while the smaller group of 4 specimens included the *C. javanus* specimens from Malaysia along with two other Malaysian samples from the BOLD database. These results clarified that *C. vitellinus* was a distinct species and not a synonym of *C. javanus.*

Attempts to examine the polytene chromosomes of these species were only partly successful. Specimens of *C. vitellinus* from Australia reared in the laboratory at 20°C produced chromosomes from which it was possible to determine that there were four chromosomes with the thummi-chromosome arm combination (AB, CD, EF, G) with a nucleolus subterminal on arm G and a Balbiani ring about a third from the other end (Fig. 4). For *C. javanus* it was only possible to determine that there were four polytene chromosomes, possibly with the same arm combination as *C. vitellinus.*

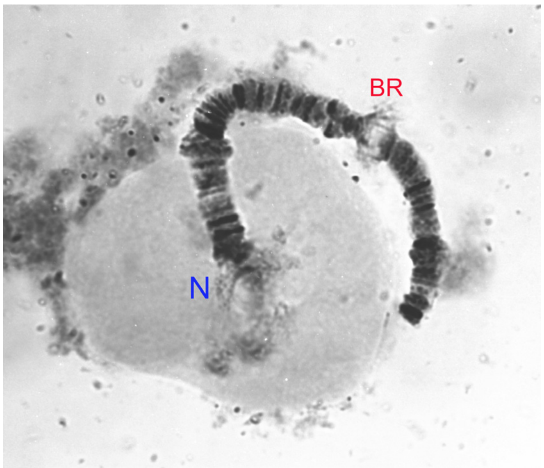

Figure 4. Arm G of *Chironomus vitellinus*: N- nucleolus; BR – Balbiani ring.

Discussion

Chironomus javanus and *C. vitellinus*

It is clear that all but three of the previous publications on *C. javanus* have in fact dealt with *C. vitellinus.* The problem appears to have arisen from the important publication on the Chironomidae of Micronesia by Tokunaga (1964), who may not have been aware of the recent description of *C. vitellinus* by Freeman (1962), as he does not cite this paper. However, since Freeman did not illustrate or mention the TIX setae in his description, Tokunaga as well as some other authors who did cite the Freeman paper, would not have immediately recognized it as a different species because only the small expansion of the end of the anal point of *C. vitellinus* is shown. Hashimoto et al. (1981) quote the paper but not in relation to *C. vitellinus* and Sasa and Hasagawa (1983) cite Freeman in their references but do not actually refer to it in the text. Al Shami et al. (2012) quote Johannsen (1932) and identify their specimens as *C. javanus.* However, they clearly state that the larvae had 7

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vitellinus   A T A T A A/G A C T C T T A/G A T T C C/T C T A T AT C T TC T C/T T T A TC  T T G C T A T T A G A T C
javanus   3' T A G A T  T    C T C T A C  C  G A A T  A   T A T A TC T A AT A  A  A A T AT A A A C A G C G G A T C T 5'
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Figure 3. Alignment of the COI barcode base pairs that differ between *C. vitellinus* (above) and *C. javanus* (below).

teeth on the premandible and illustrate the patch of setae on TIX of the adult male, so have clearly actually re-described *C. vitellinus.* They apparently did not recognize the small number of true *C. javanus* in their samples but the adult sent to JM was so deformed they may not have been able to identify it. The larvae would not have appeared different if the premandibles were not lying in optimal orientation, and other recognized differences are minor differences in the relative sizes of the anal and ventral tubules (dorsal pair of anal tubules (AT) longer and ventral tubules (VT) of equal length in *C. vitellinus,* ventral pair of AT longer and thinner and anterior pair of VT longer with the two pairs of VT arising close together (Chaudhuri et al. 1992) in *C. javanus*; other characters may differ in mean values but with considerable overlap of individual ranges.

These results clearly indicate that the prevailing view that *C. javanus* is a widely distributed species and *C. vitellinus* a possible synonym is incorrect. Instead they are separate, but closely related species, with *C. vitellinus* widely distributed at least from Africa eastward to Micronesia and in new records reported here, southeastern North America and in Puerto Rico (Fig. 5), while *C. javanus* appears to be restricted to a small area around India and Malaysia, often in common with *C. vitellinus.* In this regard it can be noted that *C. daitocedius* Sasa and Suzuki (2001) is a synonym of *C. vitellinus,* not *C. javanus* as stated by Yamamoto and Yamamoto (2018).

Chironomus vitellinus has a short development time: Reyes-Maldonado et al. (2021) reared the larvae at 27^{O}C and found that adult males emerged after 10 days and females about 2 days later. In the wild, the species breeds in a number of different habitats – commonly in rice paddies (e.g. – in Asia (Al Sharmi et al., 2012), Australia and Papua New Guinea); in tsunami-affected coastal pools in Thailand (Cranston 2007); as well as artificial containers such as a 44 gallon drum (Papua New Guinea) or dark colored containers, bird baths and water troughs in Puerto Rico (Reyes-Maldonado et al*.,* 2021). The short development time would be advantageous in completing development before a temporary habitat dried out, as has also been reported for other species utilising such habitats (e.g. McLachlan and Cantrell, 1980; Nolte 1995).

There are some minor differences between specimens from different locations, probably reflecting times of separation and differential selection pressures, particularly in the Americas where there other species with similar ecology. The most notable difference is the coloration of the male abdomen, which is usually yellowish or greenish and lacking dark markings, while Florida and Puerto Rico specimens have a green abdomen with a light quadrilateral patch on tergites II-VI, with TVIII totally dark. In view of the suggestion that these populations may have derived from Japan, it may be noted that the Japanese specimens have a green abdomen, although lacking dark markings (Hashimoto 1984).

Chironomus sp. "Florida"

Epler (2001) keyed and illustrated an unusual *Chironomus* larva with a multitoothed premandible from Florida, calling it *Chironomus* sp. "Florida".

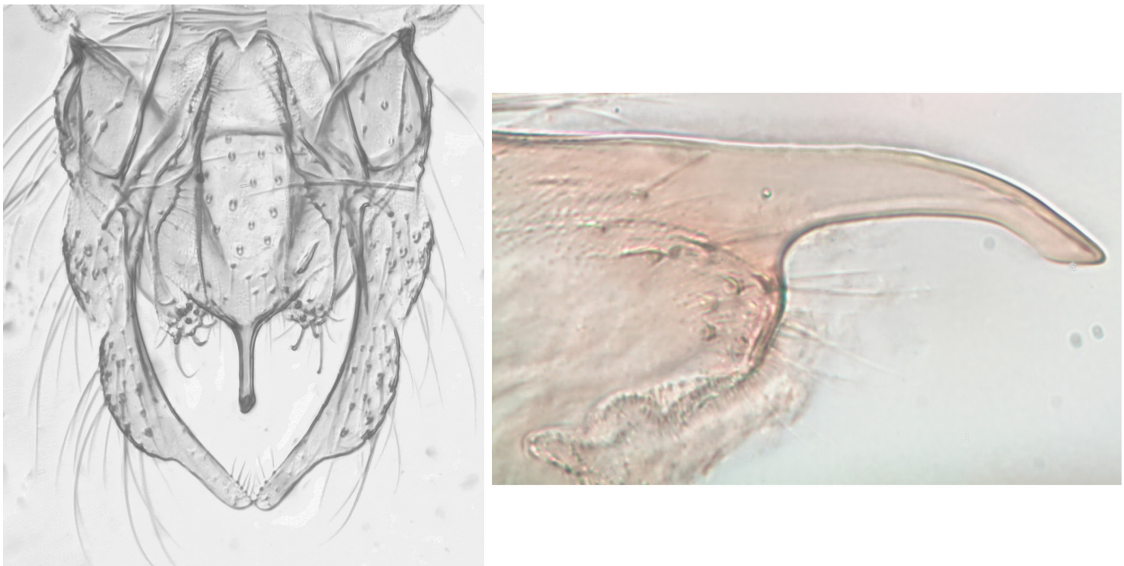

Figure 5. Male hypopygium (left) and anal point (right) of Florida specimen of *Chironomus vitellinus.*

When he reared *Chironomus* larvae from a birdbath at his house in northern Florida in 2013 and 2017, he noted that the larvae had multitoothed premandibles and thought he had reared *C*. sp. “Florida” larvae. However, lengthy correspondence between the authors resulted in an identification of *Chironomus vitellinus* for these birdbath larvae, as well as those reared from birdbaths and other outdoor containers by Bob Rutter in Port Charlotte, FL, Doug Strom in Gainesville, FL, and Alonzo Ramirez from several localities in Puerto Rico.

As well, in 2018, Alonzo Ramirez contacted JHE to identify the *Chironomus* he was breeding in a lab culture, collected from various sites in Puerto Rico. JHE examined larvae, pupae and adults and determined they were the same species he had reared from his birdbaths, but at this stage he still thought his birdbath larvae represented *C*. sp. “Florida”, and provided this incorrect identification that was subsequently used in Reyes-Maldonado et al. (2021). As noted above, it was after this that the identity of the birdbath larvae was revealed to be *C. vitellinus*.

Thus one mystery was cleared up – but what about *C*. sp. “Florida”? This taxon was initially known from the single larva described by Epler (2001) but was a bit different from the new birdbath larvae, perhaps due to a worn mentum and premandibles.

With the exception of the unusual mentum and premandible, *C*. sp. “Florida” is a rather typical *Chironomus* larva, including the presence of furrows at the base of the mandible. The single, apparently mature, larva in JHE’s collection is about 12 mm long, and has two pairs of long, straight ventral tubules and a pair of long lateral tubules, i.e. a melanotus-type larva (Proulx et al. 2013). No other life stages are known.

Larvae of *C. vitellinus* and *C*. sp. “Florida” are easily separated by the mentum and premandibles (Figs 6-7). *Chironomus vitellinus* has a typical *Chironomus* mentum with a trifid median tooth (Hashimoto 1984 and Fig. 6) arising lower than the lateral teeth; the mentum of *C*. sp. “Florida” has a simple rounded median tooth (all the teeth of the mentum are rounded) and arise at the same level as the other teeth (Fig. 7).

At first glance, the mentum of *C*. sp. “Florida” (Fig. 7) looks more like that of some *Polypedilum* (e.g. Fig. 10.58 of Pinder and Reiss (1983).

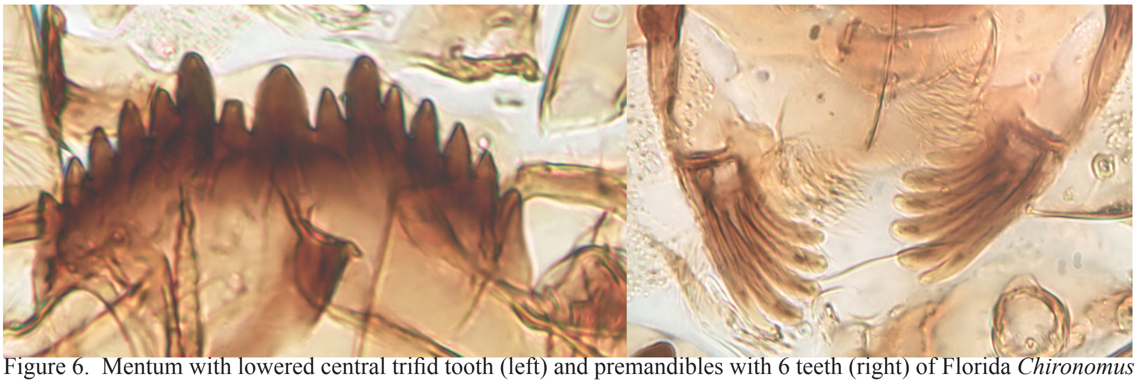

Figure 6. Mentum with lowered central trifid tooth (left) and premandibles with 6 teeth (right) of Florida *Chironomus vitellinus*.

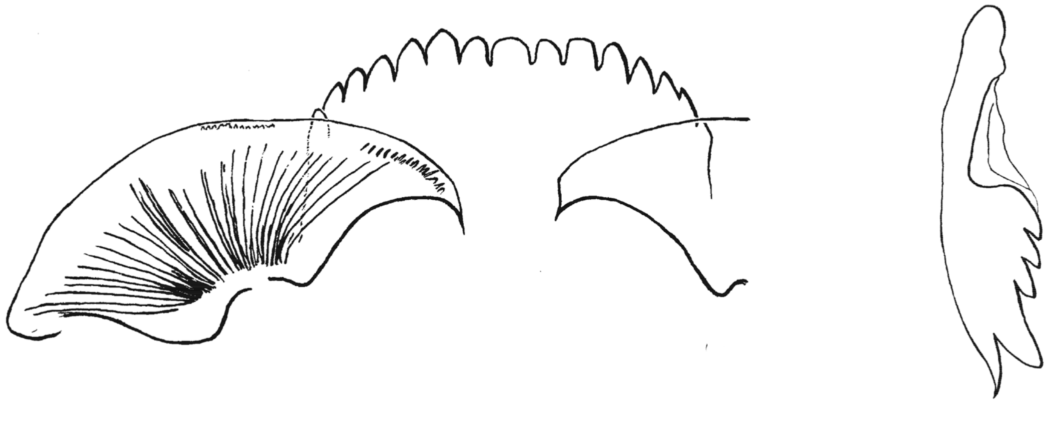

Figure 7. Mentum and ventromental plate (left) and premandible with 5 teeth (right) of *Chironomus* sp. “Florida”.

The premandible of *C. vitellinus* usually has 6 well defined teeth (Fig. 6) with a 7th tooth barely indicated in most populations, while the premandible of *C.* sp. "Florida" has 5 teeth (Fig. 7). The premandibular teeth of *C. vitellinus* are more widespread and fan-like in most mounts, with 6 long teeth, very similar to those of *Kiefferulus* Goetghebuer species; those of *C.* sp. "Florida" are shorter and wider, and quickly become progressively shorter (Fig. 7). An early instar larva of C. sp. "Florida" appears to have only 4 premandibular teeth.

The original *C.* sp. "Florida" larvae that Epler (2001) examined were collected in south Florida by Bob Rutter; he found larvae in burrows made in the water lily *Nuphar* Sm. (Nymphaeceae) by the aquatic larva of the noctuid moth *Bellura* Walker. More recently, Doug Strom (pers. comm.) looked at *Nuphar* from two Florida lakes and collected several larvae but was unsuccessful in rearing them. Perhaps the unusual mentum is an adaptation to life within aquatic moth larvae tunnels. The taxon remains an enigma.

Chironomus vitellinus and *C.* sp. "Florida" are not unique among *Chironomus* in having premandibles with multiple teeth as *C. okinawanus* Hasegawa & Sasa 1987 is widespread in China (Hongqu Tang, pers. comm.) and several species occur in South America (Reiss, 1974; Correia and Trivinho-Strixino, 2007). However these species are not conspecific with *C. vitellinus* or *C.* sp. "Florida" as they differ in adult and/or larval morphology, including the morphology and tooth number of the larval premandible.

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