

A revision of genus *Toxorhynchites* Theobald, 1901, in the South-East Asian countries, with description of a new species *Toxorhynchites (Toxorhynchites) darjeelingensis* from West Bengal, India (Diptera, Culicidae)

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Abstract

The genus *Toxorhynchites* (Tribe Toxorhynchitini), comprising 89 species worldwide which are organized under four subgenera (*Afrorhynchus*, 19 species; *Ankylorhynchus*, 4 species; *Lynchiella*, 16 species and *Toxorhynchites*, 50 species), is characterized by mosquitoes that do not feed on blood; instead they subsist on variety of plant juices and have their mouth parts commensurately designed. Globally there are about 3,543 species of mosquitoes (Family: Culicidae), of which nearly 3,061 species are culicines under Subfamily Culicinae that is further divided into eleven tribes one of which is Toxorhynchitini Lahille, 1904, represented by a solitary genus *Toxorhynchites* Theobald, 1901. Species of the subgenus *Toxorhynchites* alone are prevalent in the southeastern Asian countries (Indonesia, 12 species; India, 9 species; Thailand, 8 species; Bangladesh, 2 species; Sri Lanka, 2 species; DPR Korea, 1 species; Myanmar, 1 species; and Nepal, 1 species). A taxonomic comparison is made amongst all taxa endemic to these countries. Ironically no species of *Toxorhynchites* has ever been reported from Bhutan, Maldives and Timor-Leste. *Toxorhynchites (Tox.) splendens* is the most common species amongst all and has so far been recorded from only seven countries including India which is a home for as many as ten species, including the current *Tx. (Tox.) darjeelingensis* sp. n. collected from the foothills of Darjeeling Himalayan Mountains in the West Bengal State. *Toxorhynchites (Tox.) darjeelingensis* sp. n. is described, with a comparison offered with its closest allies, i.e., *Tx. bengalensis*, *Tx. splendens* and *Tx. tyagii*.

Keywords: *Toxorhynchites darjeelingensis* sp. n., Culicinae, mosquitoes, Southeast Asia.

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Introduction

Mosquitoes (Family Culicidae; Order Diptera) are highly diverse creatures, represented by a monophyletic taxon (Wood and Borkent, 1989; Miller *et al.*, 1997; Harbach and Kitching, 1998). Culicidae is a large and abundant group of strikingly varied species (3,543) (Harbach and Howard, 2007; Tyagi *et al.*, 2015) that occur throughout tropical and temperate regions of the world, and well beyond the Arctic Circle. Most of the mosquito species of the world (3,061) belong to subfamily Culicinae which is subdivided into 11 tribes, including Toxorhynchitini that is represented by single genus, *Toxorhynchites*. The species under Toxorhynchitini, characterized by an evenly

rounded scutellum, make a very special group of mosquitoes, next to Anophelini in the evolutionary tree, which are generally large sized mosquitoes and essentially vegetarian, deriving their nutrition requirements from plants only with an exquisitely designed mouthparts for the purpose. The genus comprises 89 species which are organized under four subgenera, namely, *Afrorhynchus* (19 species), *Ankylorhynchus* (4 species), *Lynchiella* (16 species), and *Toxorhynchites* (50 species). *Toxorhynchites* species are endemic to Asia, and the southeast region comprising eleven countries, where as many as 24 species occur (Tyagi *et al.*, 2015). The larvae are predatory and opportunistically feed

on other mosquito larvae, and thus have the potential of a biological agent for controlling obnoxious mosquitoes particularly the dengue/chikungunya vectors, *Aedes aegypti* and *Ae. albopictus* (Collins and Blackwell, 2000).

Genus *Toxorhynchites* Theobald, 1901

Adults of *Toxorhynchites* species are large and colourful, with their body covered with green, purple or red iridescent scales. The distal half of the proboscis is slender and bent sharply downwards and backwards. The scutellum is evenly rounded (as in *Anopheles* and *Bironella*) and the posterior margin of the wing is distinctly emarginated opposite the termination of vein CuA. Larvae have mouth brushes composed of about 10 broad, flat filaments that are used to capture prey, and the dorsal and ventral abdominal setae occur in groups and large sclerotized plates. The comb and pecten are both absent. The *Toxorhynchites* larvae are found in plant cavities, mainly tree holes and bamboo stumps, but sometimes in littered tin boxes as well as earthen pots and even physiologically active phytotelmata such as insectivorous pitcher plants, like *Nepenthes* species (Tsukamoto, 1989), although Munirathinam *et al.* (2014) who reported 128 species belonging to all the three tribes, Anophelini, Toxorhynchitini and Culicini, from a variety of plant materials in certain regions of Western Ghats, did not report such a breeding behavior of a *Toxorhynchites* species (Table 1). The larvae of all species are predators. They feed mainly on other mosquito larvae, including their own kind when other species are few or absent. The adults are basically forest mosquitoes. Male and female both feed exclusively on nectar and sugary substances. Due to non-blood sucking behaviour, they are not of medical importance. However, the larvae of a few species have been used with some success to control medically important mosquitoes whose immature co-inhabit with that of *Toxorhynchites* in plant cavities and artificial containers.

Subgenus *Toxorhynchites* Theobald, 1901

(1) Distribution of species:

The subgenus *Toxorhynchites*, comprising

24 species disseminated over eight countries, viz., Indonesia (12), India (9), Thailand (8), Bangladesh (2), Sri Lanka (2), DPR Korea (1), Myanmar (1) and Nepal (1), is endemic to the south-east Asian countries (Table 2). Ironically, Bhutan, Maldives and Timor-Leste are yet to record any species despite abundant sylvatic environment.

(ii) Taxonomic characteristics

The subgenus *Toxorhynchites* is one of the four subgenera grouped under the genus by the same name. It is characteristically endemic to south-east Asian countries, albeit hitherto unreported from Bhutan, Maldives and Timor-Leste. Indonesia leads with species diversity enlisting 12 species, followed by India boasting of ten species, two of which having been recently discovered by the scientists of Centre for Research in Medical Entomology. Some of the salient and distinguishing subgeneric characters are offered in Table 3, whereas the species-wise distinguishing characteristics have been given in Table 4.

Identification Key to the Adults of *Toxorhynchites* Species

Adults of species under the subgenus *Toxorhynchites*, species of which are organized under seven groups, can be distinguished from those of other subgenera in the following key characteristics:

1. Tarsi entirely dark; small and slender species.....***Tx. minimus* (Theobald)**
 - Some tarsal segments with white markings; large species.....2
- 2(1)Abdominal tergites VI-VIII with lateral tufts3
 - Abdominal tergites VI-VIII weakly or without lateral tufts.....17
- 3(2)Mesonotum with conspicuous border of white or pale golden scales usually extending over the wing roots.....4
 - Mesonotum without conspicuous border of pale scales.....11
- 4(3)Proboscis with distinct median pale band or with dorsomedian pale spot.....5
 - Proboscis dark.....7
- 5(4)Proboscis with distinct median pale band; lateral tufts on tergum VI with golden

- scales, VII & VIII with brilliant orange scales.....***Tx. sunthorni* Thurman**
- Proboscis with dorsomedian pale spot.....6
- 6(5) Lateral tufts on abdominal segment VII dark with bluish black and VIII with dark golden setae.....***Tx. bickleyi* Thurman**
- Proboscis dark with few pale scales dorsally at base; Lateral tufts on abdominal segments VI & VIII with golden and VII black setae.....***Tx. speciosus* (Skuse)**
- 7(4) Each abdominal segment with two creamy yellow bands (one broad and another thin line); lateral tufts on tergum VI-VIII orange and black; Knee spots of all legs peacock blue.....***Tx. quasiferox* (Leicester)**
- Without this combination.....8
- 8(7) Mid and hind tarsomeres 3-5 complete white or dark scales.....9
- Mid tarsomeres 2-4 white; tarsi 5 dark scales; lateral tufts on tergum VI & VIII orange and VII dark setae.....***Tx. manopi* Thurman**
- 9(8) Midtarsomeres 3-5 complete white; abdominal segments III & V with incomplete medial pale bands; lateral tufts on tergum VI pale yellow; VII golden & VIII orange setae.....***Tx. edwardsi* (Barraud)**
- Mid tarsomeres 3-5 dark scales.....10
- 10(9) Lateral tufts on tergum VI & VII black & VIII with orange setae.....***Tx. tyagii* Krishnamoorthy et al.**
- Lateral tufts on tergum VI with three-fourth golden yellow one-fourth black setae, VII deep blue green and VIII pale yellow setae.....***Tx. darjeelingensis* Tyagi et al.**
- 11(3) Lateral tufts present on tergum VII & VIII and no tufts on tergum VI.....12
- Lateral tufts present on tergum VI-VIII.....13
- 12(11) Lateral tufts on tergum VII & VIII orange setae; mid tarsomeres 2 & 4 basal half white and tarsi 3 complete white scales.....***Tx. sumatranus* (Brug)**
- Lateral tufts on tergum VII & VIII black setae; mesonotum with narrow broad decumbent greenish scales becoming broader and bluish laterally.....***Tx. amboinensis* (Doleschall)**
- 13(11) Lateral tufts on tergum VI & VII with orange (or) dark brown setae.....14
- Lateral tufts on tergum VI-VIII with black and orange (or) white and black setae.....15
- 14(13) Lateral tufts on tergum VI -VIII with orange setae; fore and hind tarsi 3-5 black; mid tarsi 2-4 white and mid tarsi 5 black scales...***Tx. auranticauda* Lane**
- Lateral tufts on tergum VI white and dark brown and VII & VIII dark brown setae; mid tarsi 1-5 with white banding on basal half.....***Tx. bengalensis* Rosenberg and Evenhuis**
- 15(13) Lateral tufts on tergum VI white, VII & VIII black setae; tarsomere 5 of all legs entirely dark scales; all femora have three rows of short black spines.....***Tx. magnificus* (Leicester)**
- Lateral tufts on tergum VI-VIII with black and orange setae.....16
- 16(15) Lateral tufts on tergum VI & VII black setae; VIII orange setae; sub basal on mid tarsomere 1 with one-fourth and 2 with half white scales.....***Tx. inornatus* (Walker)**
- Lateral tufts on tergum VI with yellow and black setae; VII with black and VIII with orange setae; mid tarsomeres 1-5 with white scales; fore and hind tarsomeres 3-5 with dark scales.....***Tx. splendens* (Wiedemann)**
- 17(2) Lateral tufts on tergum VI-VIII weakly developed; VI & VIII with pale yellow and VII with black setae; tarsomeres 5 of all legs with pale and dark scales.....***Tx. albipes* (Edwards)**
- Lateral tufts on tergum VI-VIII without tufts; proboscis dark or with pale band.....18
- 18(17) Proboscis with brown scales apically and violet tinge on basal part; a ring of silvery scales at the site of the bent.....***Tx. christophi* (Portschinsky)**
- Proboscis without pale ring.....19
- 19(18) Abdominal tergum with complete or incomplete bands.....20
- Abdominal tergite V-VII with narrow incomplete basal pale bands; fore and mid tarsomeres 2-4 with pale scales.....***Tx. graveleyi* (Edwards)**
- 20(19) Abdominal tergites all with basal bands.....21

- Abdominal tergites few (II – VI) with basal bands.....24
- 21(20) First joints of palpi a little shorter; third a little longer than second; venter of abdomen without median purple strip.....***Tx. klossi* (Edwards)**
- Abdominal tergites with narrow blue or honey yellow bands.....22
- 22(21) Abdominal tergites each with narrow basal blue band; sub-basal white ring on tarsal segment 1 of all legs23
- Abdominal tergites each with rose purple scales, banded with honey yellow expanding laterally into triangular patches; mid and hind tarsomeres dark covered with brilliant metallic scales.....
.....***Tx. metallicus* Leicester**
- 23(22) Mid tarsomeres 2-5 white scales; sternite IV with large median purple spot.....***Tx. leicesteri* Theobald**
- Mid tarsi 4 and large part of 5 white scales; sternite IV with purple scales in middle.....***Tx. kempi* (Edwards)**
- 24(20) Abdominal tergites II-VI with small lateral yellowish scales; tergite I with deep blue scales dorsally; sternites IV yellowish scales interrupted medially by purple scales; ocular setae (4pairs) amber to brownish; basal half of mid tarsi 1 & 2 with white band.....
.....***Tx. acaudatus* (Leicester)**
- Abdominal tergites II-V with small lateral white scales; tergite I with golden scales dorsally; sternites IV with silvery white scales interrupted medially by brownish scales; ocular setae (3pairs) dark brown; basal one-fourth of mid tarsi 1 & 2 with white band.....***Tx. coeruleus* (Brug)**

***Toxorhynchites (Toxorhynchites) darjeelingensis* Tyagi *et al.*, sp. n.**

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Materials and Methods

Larvae and pupae of *Tx. (Tox.) darjeelingensis*, sp. n. were collected from Ghadhalar Kurthi, Matha Bhanga block, Cooch Behar district in the foothills of Darjeeling mountains (West Bengal, India), during May-June 2012. Specimens were collected from littered battery chambers,

having a capacity of 4 lit., at an altitude of 100-150m. Specimens were individually reared to the adult stage for species identification, using keys by Barraud (1934). Chaetotaxy of the associated larval and pupal exuviae were examined following Harbach and Knight (1980).

Three legs from one side of one paratype specimen were used for molecular analysis. From the homogenized material a whole genomic DNA was extracted following the techniques described by Dhananjeyan *et al.* (2010). The genomic DNA isolated was used to amplify the mitochondrial Cytochrome C Oxidase subunit I (COI) gene following Simon *et al.* (1994). The amplified PCR product was visualized on a 1.2% agarose gel using a gel documentation system (Vilber Lourmet, France) (Fig. 1). The product was sequenced commercially (Eurofins India Pvt. Ltd., 183, Gayathri Tech Park, EPIP – II Phase Whitefield, Bangalore-560066, Karnataka, India).

The nomenclature and chaetotaxy used in the description of new species, *Toxorhynchites (Tox.) darjeelingensis*, were described following Harbach and Knight (1980, 1982) and Bickley and Ward (1989).

Description

Female: *Wing:* 5.7mm, *proboscis* 5.9mm, *fore femur* 4.1mm, *abdomen* 5.3mm. ***Head:*** (Fig. 2): Integument blackish, scales of vertex light brown and with broad distinct violet orbital line; *proboscis* bluish; *maxillary palpus* bluish purple, scattered pale scales on dorsum, comprised of 4 palpomeres with equal length; *antennal pedicel* with a large conspicuous dense patch of silvery scales, scales of *flagellomeres* 1–6 dense with many small hairs. ***Thorax*** (Fig. 2): Integument dark brown or blackish, *mesonotum* densely covered with rather dull bronzy scales with bluish-green tinge, scales slightly narrower on disc than on sides, with whitish yellow scales with blue tinge patches over wing root to scutellum; *antepnotum* with 6-8 minute hairs along with bluish scales, *postpronotum* with silvery scales along with 4-5 setae, *pleural* and *coxal* scales silvery; one weak lower mesepimeral seta and usual row of caudal mesepimeral setae. ***Abdomen*** (Fig. 3): Terga largely bluish or greenish, tergum I with blue-green scales in the middle and brownish yellow scales laterally; tergum II with blue-green scales in

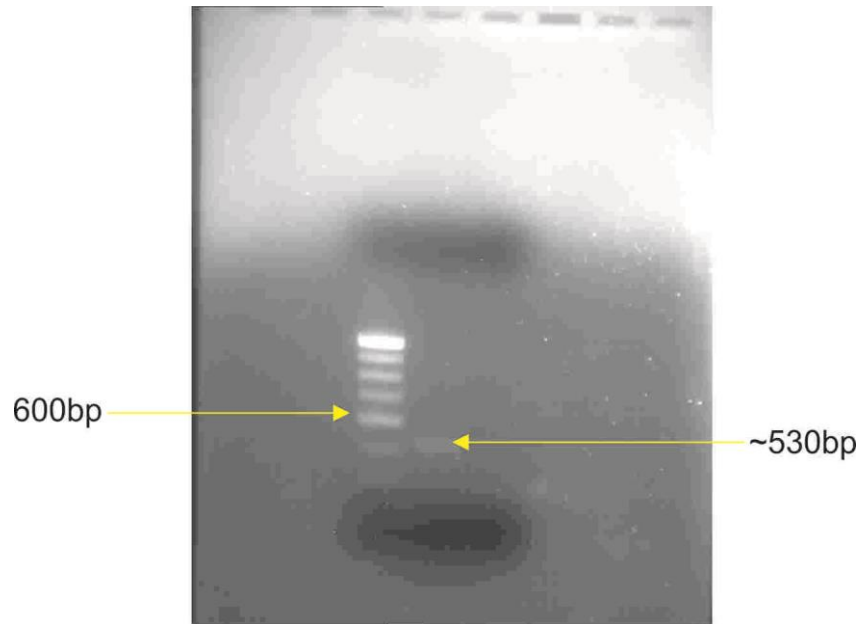


Figure 1. Gel picture showing the amplification of ~530bp amplicon of *Tx. darjeelingensis* sp. n.

the middle and golden scales laterally; terga III & V with deep green scales in center and apico-lateral broad golden scales visible from above; tergum IV with deep-green scales; with 75% of golden yellow and 25% of black lateral hair-tufts; tergum VII with 90% deep blue-green and remaining yellow hair tufts; tergum VIII with a bunch of pale yellow setae; sterna I & II completely with broad white scales; sternum III mainly broad white scales with narrow median dark line; sternum IV completely broad black-scaled; creamy white scales are present in major area in sternum V and VI along with black scales forming a 'V' shape medially; sternum VII with black scales forming narrow line in the median, remaining area with broad white scales; sternum VIII mainly black-scaled with few scattered white scales, and with golden yellow tuft. *Legs* (Fig. 4): Fore femur with wide basal yellow ring, mid femur black with pale scales, hind femur 70% with golden yellow, remaining black; fore and hind tibiae dark, mid-tibia with white longitudinal stripe; fore tarsomere 1 mainly pale, narrowly dark at base, fore tarsomere 2 with $\frac{1}{3}$ white basally; mid tarsomere 1 with broad basal pale band, mid tarsomere 2

entirely pale; hind tarsomere 1 with $\frac{1}{4}$ whitish yellow basal band, hind tarsomere 2 with broad basal pale band, tarsomere 3–5 of all legs dark.

Male: Generally similar to female. *Head:* Integument blackish; maxillary palpus slightly longer than proboscis; antenna verticillate, flagellomeres 1 with few white scales and numerous black scales. *Legs:* All tibia dark; fore tarsus completely dark; mid femora with a longitudinal pale line, mid tarsomere 1,2 with basal pale band and 3-5 completely dark; hind tarsomere 1 with few scattered white scales posteriorly, 2 with broad white band and 3-5 completely dark. *Genitalia* (Fig. 5): Gonocoxite 0.55 mm, gonostylus 0.52 mm, gonostylar claw 0.05 mm. Gonocoxite with numerous microsetae; gonostylus with a single sub apical gonostylar claw; medial margin of gonostylus with about 14 micro-setae distributed evenly from base to apex. Basal mesal lobe (BML) with one stout and long seta length 0.30 mm, medial surface with numerous short simple setae, less than half length of longest. Tergum IX with about 25 simple setae arranged on dorsolateral and lateral margins.

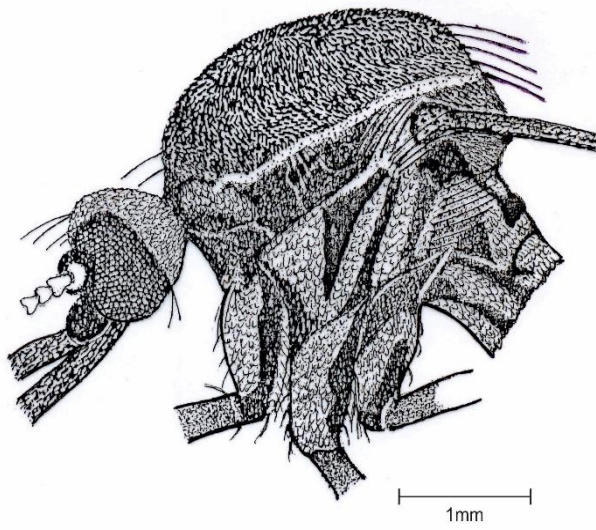


Figure 2. Head and thorax of holotype female of *Toxorhynchites darjeelingensis*, sp.n.

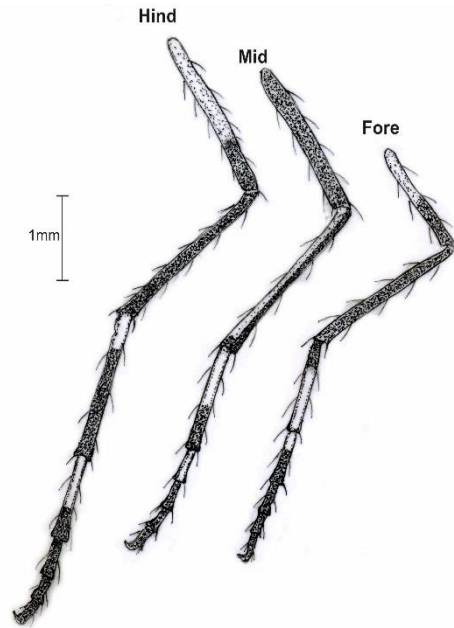


Figure 4. Legs of *Toxorhynchites darjeelingensis*, sp.n.

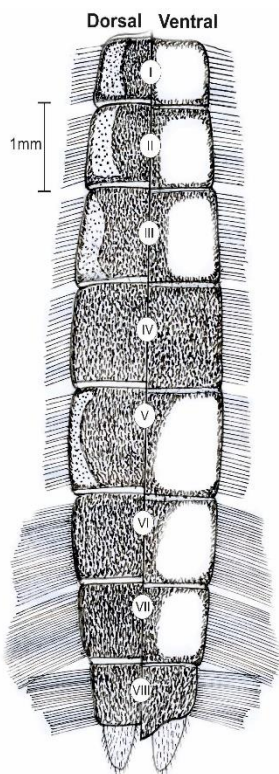


Figure 3. Abdomen of *Toxorhynchites darjeelingensis*, sp.n.

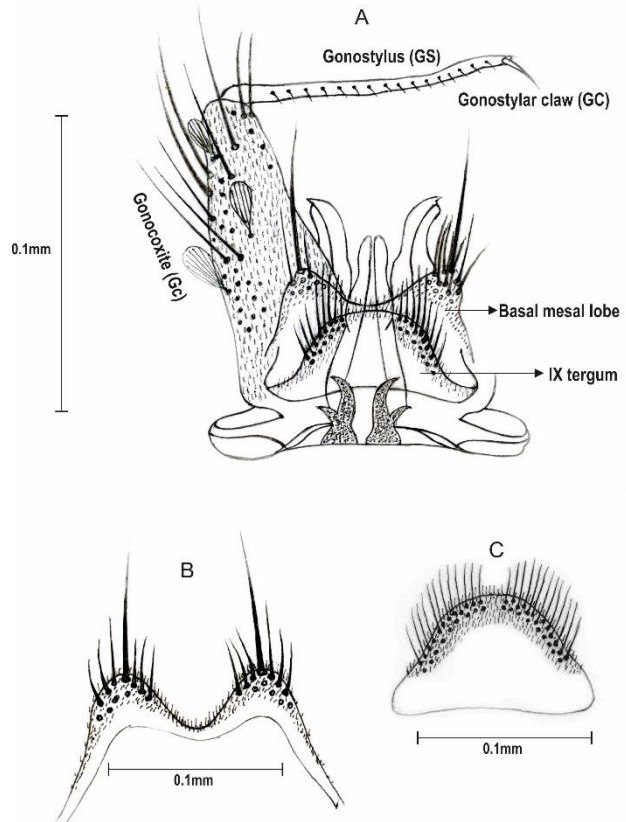


Figure 5. Male genitalia of *Toxorhynchites darjeelingensis*, sp.n. A. Dorsal aspect; B. basal mesal lobe, C. tergum IX

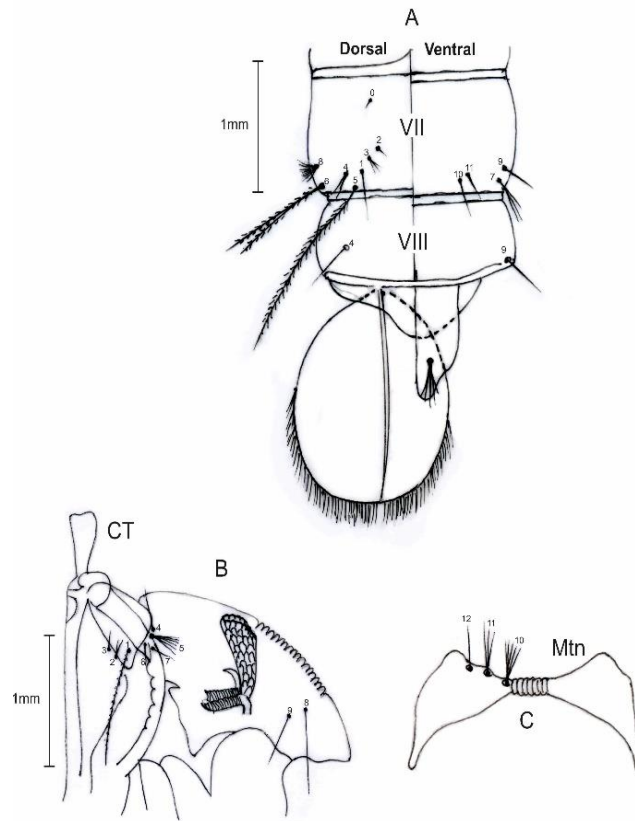


Figure 6. Pupa of *Toxorhynchites darjeelingensis*, sp.n.
A, terminal abdominal segments (VII & VIII); B, cephalothorax (CT); C, metanotum (Mtn)

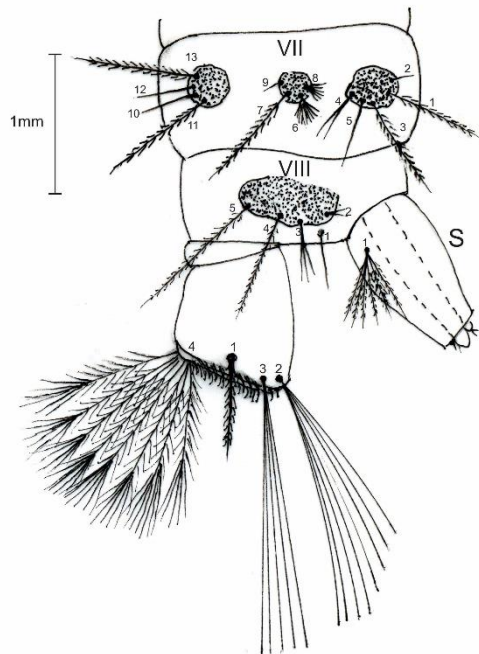


Figure 7. Terminal abdominal segments of the larva of *Toxorhynchites darjeelingensis*, sp.n.

Pupa: Abdomen 5.1 mm, trumpet 0.9 mm, paddle 1.4 mm. Chaetotaxy as an illustrated (Fig.6), and the range of variation shown in Table 5. **Cephalothorax:** Moderately pigmented; seta 1-CT single, very long, barbed; seta 2-CT with 2 branched; setae 3-4, 6-9-CT single, seta 5-CT with 5-7(7) branches; seta 10-CT with 4,5 (5) branches, seta 11-CT with 3,4 (3) branches and seta 12-CT with 1,2 (1) branches. **Trumpet:** Dark orange-brown, heavily pigmented, almost twice as long as wide at apex. **Abdomen:** Bright brown, moderately pigmented, large setae darker than integument; seta 6-VII single, bifid at the center; seta 7-VII single, forked at the center; paddle pigmented, more or less rounded, very wide, width about 0.65 of length, and similar as in *Tx. tyagii*, midrib complete, distal 0.57 of outer and inner margins with long fine hair-like spicules; setae 1,2-Pa absent.

Larva: Head 1.21 mm, siphon 0.87 mm, saddle 0.76 mm. Chaetotaxy as illustrated

(Fig.7) and the range of variation shown in Table 6. **Antenna:** Concolorous with head capsules. **Thorax:** Setae, tubercles and plates strongly pigmented; seta 7-P,T double, barbed, stiff; seta 10-P,M,T single, thin, barbed; seta 13-M with 2 branches, stiff and barbed. **Abdomen:** Setae 10,12,13-I on single plate, seta 11-I on separate plate; seta 3-I slightly longer than setae 1- I and 4-I; seta 3-II double, barbed, 3-III-V single, long, barbed; setae 6-II-V and 7-I –IV double, long, barbed; setae 1,3-VII long, strongly barbed; seta 1-VIII distinctly separate from large sclerotized plate and without tubercle; seta 2-VIII simple with two branches; seta 3-VIII origin with single at the end with three branches; setae 4,5-VIII single, long, barbed. **Siphon:** Index about 1.68; seta 1-S with 6 branched. **Segment X:** Uniformly darkly pigmented; saddle with long spicules on caudolateral margins; ventral brush (seta 4-X) with 8 pairs of setae.

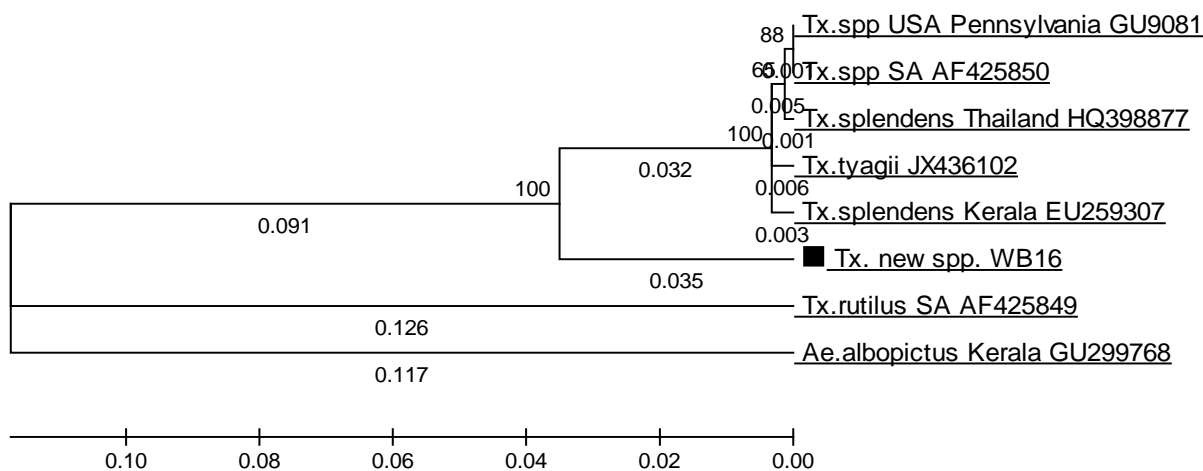


Fig. 8. Phylogenetic tree showing the positioning of *Toxorhynchites darjeelingensis* sp. n. (WB16) along with other associated taxa of the genus *Toxorhynchites*

Molecular characterization

For phylogenetic analysis mitochondrial Cytochrome C Oxidase subunit I gene sequences of six species of *Toxorhynchites* were included that were retrieved from GenBank. The GenBank Accession Numbers of each sequence is shown in the phyletic tree. Of the six sequences utilized for the phylogenetic tree construction, 2 sequences belonged to *Tx. splendens* from Thailand (HQ398877) and India (EU259307). Two sequences of *Toxorhynchites* genus, which are

not identified to species level; one collected in Pennsylvania, USA (GU908123) and another collected in South Africa (AF425850). The fifth CO1 gene sequence belong to *Tx. tyagii* (JX436102), isolated in Nilgiri hills, Tamil Nadu, India and identified and reported as a new species by CRME, India (Krishnamoorthy *et al.*, 2013). The 6th sequence is of *Tx. rutilus* (AF425849) from South Africa. The COI gene sequence of *Aedes albopictus* (GU299768) from Kerala, India has been included in the

Phylogenetic tree construction as an outgroup.

The evolutionary history of *Toxorhynchites darjeelingensis* sp. n. (WB16) was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

The Phylogenetic tree was linearized assuming equal evolutionary rates in all lineages (Tekezaki *et al.*, 2004). The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 392 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 software (Tamura *et al.*, 2007).

Type material: Holotype female (coded A#1793) with associated larval (Le#851) and pupal (Pe#829) exuviae mounted on microscopic slides with the following collection data: INDIA, West Bengal, Jalpaiguri district, Darjeeling hills, 21st May 2012, collected as larva from littered battery chambers at an altitude of 150m, collected by R. Govindarajan, deposited in the CRME Museum, ICMR, Madurai, Tamil Nadu, India. The allotype male (coded A#1794) with associated larval (Le#852) and pupal (Pe#830) exuviae, and 2 paratype males with Le and Pe mounted on microscopic slides, have the collection data same as that of the holotype. All are also deposited in the CRME Museum.

Distribution: Known only from the type locality in Jalpaiguri district, West Bengal, India.

Bionomics: The immature stages of *Tx. darjeelingensis* sp. n. were collected in a littered battery chambers, having a capacity of

4 lit., at an altitude of 100-150m from Ghadhalar Kurthi, Matha Bhanga block, Cooch Behar district in the foothills of Darjeeling mountains (West Bengal, India) in May-June, 2012. Larvae of *Tx. darjeelingensis* were found in association with *Armigeres (Leicesteria) magnus*, *Stegomyia (Heteraspidion) annandalei*, *Aedes albopictus* and *Tripteroides (Rachionotomyia) aranoides*.

Etymology: This species is designated name after the place of its discovery, the Darjeeling hills in West Bengal State, India.

Discussion

Adults of *Toxorhynchites (Tox.) darjeelingensis* sp. n. are superficially similar to *Tx. (Tox.) bengalensis*, *Tx. (Tox.) splendens* and *Tx. (Tox.) tyagii*. However, the diagnostic characters for larva, pupa, adult (mesonotum, abdomen, wing, legs and male genitalia) of these species clearly distinguish *Tx. darjeelingensis* sp. n. from the rest as shown in Table 6. These distinguishing characters are summarized below:

- (i) Adult mesonotum with broad pale yellow scales over wing root to scutellum are present in *Tx. darjeelingensis* and *Tx. tyagii* but absent in *Tx. bengalensis* and *Tx. splendens*.
- (ii) The lateral tufts of VI-Te in *Tx. darjeelingensis* is two-third deep blue, with remaining black but in *Tx. tyagii* it is two-third black and remainder only is yellow; white and dark brown in *Tx. bengalensis*, and yellow and black in *Tx. splendens*.
- (iii) Adult of *Tx. darjeelingensis* is a rather small sized mosquito, next to *Tx. minimus*.
- (iv) In male genitalia, BML with one stout apical seta present in *Tx. splendens* and *Tx. darjeelingensis* compared to two stout apical seta present in *Tx. bengalensis* and *Tx. tyagii*.
- (v) The pupa of *Tx. darjeelingensis* can easily be separated on the basis of 10-C with 5 branches, whereas others have lesser (*Tx. bengalensis*) and more (*Tx. tyagii*) (cf. Table 6).
- (vi) Larva of *Tx. darjeelingensis* appears closer to *Tx. splendens* and *Tx. tyagii*, but the new species can be quickly distinguished on the basis of seta 7-M with 3 branches, and 13-M double in thoracic region.

Table 1. Preference of different *Toxorhynchites (Tox.)* species in selecting habitats for breeding in the South-East Asia Region countries

S.No	Species / Habitat	Bamboo Stump	Coconut husks	Discarded container	Discarded battery	Leaf axil	Metal barrels / tin	Mud pot	Pitcher plants	Rocky pool	Sintex Tank	Small wells	Tree hole	Resting collection
1	<i>Tx. acaudatus</i>								●					
2	<i>Tx. albipes</i>												●	
3	<i>Tx. amboinensis</i>	●												
4	<i>Tx. auranti-cauda</i>			●									●	
5	<i>Tx. bengalensis</i>	●											●	
6	<i>Tx. bickleyi</i>													●
7	<i>Tx. coeruleus</i>								●				●	
8	<i>Tx. christophi</i>												●	
9	<i>Tx. edwardsi</i>		●										●	
10	<i>Tx. gravelyi</i>	●											●	
11	<i>Tx. inornatus</i>		●	●			●		●		●	●	●	
12	<i>Tx. kempii</i>	●												
13	<i>Tx. klossi</i>												●	
14	<i>Tx. leicesteri</i>	●												
15	<i>Tx. magnificus</i>	●											●	
16	<i>Tx. manopi</i>													●
17	<i>Tx. metallicus</i>	●											●	
18	<i>Tx. minimus</i>	●											●	
19	<i>Tx. quasiferox</i>	●							●					
20	<i>Tx. speciosus</i>			●			●		●		●	●	●	
21	<i>Tx. splendens</i>	●		●		●		●		●	●		●	
22	<i>Tx. sumatranus</i>								●					
23	<i>Tx. sunthorni</i>													●
24	<i>Tx. tyagii</i>						●							
25	<i>Tx. darjeelingensis</i> sp.n.				●									

Table 2. Geographical distribution of taxa under subgenus *Toxorhynchites*

Sl. No.	Species	Country	Describing valid authority
1	<i>Tx.(Tox.) acaudatus</i>	Indonesia	Leicester, 1908
2	<i>Tx.(Tox.) albipes</i>	India, Thailand	Edwards, 1922
3	<i>Tx.(Tox.) amboinensis</i>	Indonesia	Doleschall, 1857
4	<i>Tx. (Tox.) auranticauda</i>	Indonesia	Lane, 1992
5	<i>Tx.(Tox.) bengalensis</i>	Bangladesh	Rosenberg and Evenhuis, 1985
6	<i>Tx.(Tox.) bickleyi</i>	Thailand	Thurman, 1959
7	<i>Tx. (Tox.) coeruleus</i>	Indonesia	Brug, 1934
8	<i>Tx.(Tox.) christophi</i>	DPR Korea	Portschinsky, 1884
9	<i>Tx.(Tox.) edwardsi</i>	India	Barraud, 1924
10	<i>Tx.(Tox.) gravelyi</i>	India, Thailand	Edwards, 1921
11	<i>Tx.(Tox.) inornatus</i>	Indonesia	Walker, 1865
12	<i>Tx.(Tox.) kempi</i>	India, Indonesia	Edwards, 1921
13	<i>Tx(Tox.) klossi</i>	India	Edwards, 1921
14	<i>Tx.(Tox.) leicesteri</i>	Thailand	Theobald, 1904
15	<i>Tx.(Tox.) magnificus</i>	Thailand	Leicester, 1908
16	<i>Tx.(Tox.) manopi</i>	Thailand	Thurman, 1959
17	<i>Tx(Tox.) metallicus</i>	India, Indonesia	Leicester, 1904
18	<i>Tx.(Tox.) minimus</i>	India, Indonesia and Sri Lanka	Theobald, 1905
19	<i>Tx.(Tox.) quasiferox</i>	Indonesia	Leicester, 1908
20	<i>Tx.(Tox.) speciosus</i>	Indonesia	Skuse, 1889
21	<i>Tx.(Tox.) splendens</i>	Bangladesh, India, Indonesia, Nepal Myanmar, Sri Lanka and Thailand	Wiedemann, 1819
22	<i>Tx.(Tox.) sumatranus</i>	Indonesia	Brug, 1939
23	<i>Tx.(Tox.) sunthorni</i>	Thailand	Thurman, 1959
24	<i>Tx.(Tox.) tyagii</i>	India	Krishnamoorthy <i>et al.</i> , 2013

Table 3. Characters of subgenera under *Toxorhynchites*

S. No.	<i>Afrorhynchus</i> Ribeiro	<i>Ankylorhynchus</i> Lutz	<i>Lynchiella</i> Lahille	<i>Toxorhynchites</i> Theobald
1	Mesokatepisternum with a small patch of golden scales; scales on forecoxa all or almost all golden; laterotergite with few or no scales	Mesokatepisternum without golden scales; scales on forecoxa all white; laterotergite densely clothed with scales	<i>ibid.</i>	<i>ibid.</i>
2	Male midungues small, equal and simple; gonostylus widened at middle; gonostylar claw long; dorsal bridge of aedeagus wide; paraproct appearing divided into a proximal and a distal portion by a narrow unsclerotized transverse band	Male midungues unequal, one of them toothed and stronger; gonostylar claw small; dorsal aedeagus bridge narrow; paraproct without unsclerotized transverse band	<i>ibid.</i>	<i>ibid.</i>
3	-	Female antenna subplumose, with long verticillate hairs; maxillary palpus about as long as proboscis, with 3 distinct palpomeres, of which the apical one is the longest, pointed and directed upward	<i>ibid.</i>	<i>ibid.</i>
4	-	-	Female antenna normal, not subplumose; maxillary palpus obviously shorter than proboscis	<i>ibid.</i>
5	-	-	Female maxillary palpus about 0.67 – 0.75 length of proboscis, with 3 distinct palpomeres, of which the second is the longest	<i>ibid.</i>
6	-	-	-	Female maxillary palpus about 0.25 of proboscis with only 2 distinct palpomeres and shorter

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Table 4. Important characters for each species

S.No	Species	Head	Thorax	Abdomen		Legs		Male genitalia
1	<i>Tx. acaudatus</i>	ocular setae 4 pairs with amber to brownish		VI-VIII with tufts	tergites II -VI with lateral yellow scales; tergite I with deep blue; sternite IV yellow scales interrupted medially by purple scales	tarsi with white markings	basal 1/2 of mid tarsi 1 & 2 with white band	Basal mesal lobe with 3 stout apical seta; medial margin of gonostylus with numerous microsetae from apex to just above middle
2	<i>Tx. albipes</i>			VI-VIII with weak tufts	VI & VIII pale yellow, VII -dark	tarsi with white markings	tarosomeres 5 of all legs pale and dark	IX-Te narrow, apical border not produced into lobes, lateral plate with few small obvious teeth; gonostylus with few microsetae
3	<i>Tx. amboinensis</i>		mesonotum without border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VI-without tuft, VII & VIII- dark	tarsi with white markings		Basal mesal lobe with 1 stout apical seta; few short mesal hairs in gonostylus from middle to apex
4	<i>Tx. auranticauda</i>		mesonotum without border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VI - VIII with orange	tarsi with white markings	midtarsi 2-4 white,t5 black	not available
5	<i>Tx. bengalensis</i>		mesonotum without border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VI- white and dark brown, VII & VIII- dark brown	tarsi with white markings		Basal mesal lobe with 2 stout apical setae; gonostylus with few microsetae restricted to apical 1/3
6	<i>Tx. bickleyi</i>	proboscis with dorsomedian pale spot	mesonotum with border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VII- dark with bluish black, VIII-dark golden	tarsi with white markings		not available
7	<i>Tx. coeruleus</i>	ocular setae 3 pairs with dark brown		VI-VIII without tufts	tergites II-V with lateral white scales; tergite I with golden scales; sternite IV with silver white scales interrupted medially by brownish scales	tarsi with white markings	basal 1/4 of mid tarsi 1 & 2 with white band	Basal mesal lobe with 2 stout apical seta; gonostylus with numerous microsetae extending from apex to near base
8	<i>Tx. christophi</i>	proboscis with a ring of silvery scales at site of bend		VI-VIII without tufts		tarsi with white markings		Basal mesal lobe with 1 stout apical seta; gonostylus without microsetae; aedeagus flask shaped; IX - Te concave, two broad projections with 17-19 hairs

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9	<i>Tx. edwardsi</i>		mesonotum with border	VI-VIII with tufts	VI- pale yellow, VII-golden, VIII - orange; III & V segment with incomplete medial pale bands	tarsi with white markings	mid tarsomeres 2-5 white	not available
10	<i>Tx. gravelyi</i>			VI-VIII without tufts	V-VII with narrow incomplete basal bands	tarsi with white markings	mid tarsomeres 2-4 pale	IX-Te with a pair of submedian lobes, more pronounced and pointed; lateral plate without obvious teeth; gonostylus with few microsetae
11	<i>Tx. inornatus</i>		mesonotum without border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VI & VII- black, VIII- orange	tarsi with white markings		Basal mesal lobe with 2-3 stout apical setae; IX-Te truncate with a pair of 12-16 bristles each; lateral plate without obvious teeth; gonostylus with numerous short microsetae from basal 1/4 to apex
12	<i>Tx. kempi</i>			VI-VIII without tufts		tarsi with white markings	mid tarsi 4 and large part of 5 white	IX-Te narrow, submedian hairy lobes not well developed with 10-12 long hairs; Basal mesal lobe with 2 stout apical setae, gonostylus with five microsetae in a row before the tip; lateral plate with a small number of minute teeth
13	<i>Tx. klossi</i>	first joints of palpi little shorter, third little longer than second		VI-VIII without tufts	abdominal tergites all with basal bands	tarsi with white markings		IX-Te broader, less emarginate, gonostylus with hair like terminal spine
14	<i>Tx. leicesteri</i>			VI-VIII without tufts	sternite IV with large median purple spot	tarsi with white markings	mid tarsomeres 2-5 white	not available
15	<i>Tx. magnificus</i>		mesonotum without border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VI -white, VII & VIII-black	tarsi with white markings	all femora with three rows of shot black spines	apparently very simple, the claspers being composed of a basal piece with simple hinged hook at the end
16	<i>Tx. manopi</i>		mesonotum with border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VI & VIII-orange, VII-dark	tarsi with white markings	mid tarsi 2-4 white, t5 dark	not available
17	<i>Tx. metallicus</i>			VI-VIII without tufts	abdominal tergites with rose purple, banded with honey yellow	tarsi with white markings	mid and hind tarsomeres dark covered with metallic scales	not available

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18	<i>Tx. minimus</i>					tarsi entirely dark		IX-Te strongly produced in middle into a shield like plate with hairs; Basal mesal lobe with 1 stout apical setae; gonostyle about length of coxite; lateral plate narrow, with few minute blunt teeth near apex
19	<i>Tx. quasiferox</i>		mesonotum with border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VI-VIII with orange and black; each segment with two creamy yellow bands	tarsi with white markings		not available
20	<i>Tx. speciosus</i>	proboscis with dorsomedian pale spot	mesonotum with border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VI- with golden, VII-dark, VIII- with golden	tarsi with white markings		not available
21	<i>Tx. splendens</i>		mesonotum without border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VI- yellow and black, VII-dark, VIII- orange	tarsi with white markings	fore and hind tarsomeres 3-5 dark	Basal mesal lobe with 1 stout apical seta; gonostylus with few microsetae restricted to distal half
22	<i>Tx. sumatranus</i>		mesonotum without border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VI- without tuft, VII-brilliant orange, VIII-brilliant orange	tarsi with white markings	midtarsi 3 complete white	not available
23	<i>Tx. sunthorni</i>	proboscis with median pale band	mesonotum with border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VI- golden scales, VII-brilliant orange, VIII-brilliant orange	tarsi with white markings		not available
24	<i>Tx. tyagii</i>		mesonotum with border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VI- yellow and black, VII-black, VIII- orange	tarsi with white markings		Basal mesal lobe with 2 stout apical seta; gonostylus with few microsetae from base to apex
25	<i>Tx. darjeelingensis</i>		mesonotum with border of broad pale yellow scales over wing roots to scutellum	VI-VIII with tufts	VI- golden yellow and black, VII-deep blue green, VIII-pale yellow	tarsi with white markings		Basal mesal lobe with 1 stout apical seta; gonostylus with numerous microsetae distributed evenly from base to apex

Table 5. Chaetotaxy of the pupa of *Toxorhynchites darjeelingensis* sp. n.

Seta No	Cephalothorax	Abdominal Segments								
		I	II	III	IV	V	VI	VII	VIII	IX
0			1	1	1	1	1	1	1	-
1	1(L,B)	m(F)	m	1-2(1) L,B	1 L,B	1 L,B	1	1	-	3-5(5)
2	2	1-3(2)	1	1	1	1	1	1	-	-
3	1	1	1	1L,Wb	2-5(4)	1,2(2)	2-4(3)	2-4(3)	-	-
4	1	2-8(5)	3-6(5)	3-7(4)	2-7(6)	4-7(5)	2-4(3)	2,3(2)	1	-
5	5-7 (6)	1-3(3)	1L,Wb	1L,Wb	1 L,B	1 L,B	1 L,B	1L,B	-	-
6	1	1L,B	1L,Wb	1L,Wb	1L,Wb	1 L,B	1 L,B	1 Bf	-	-
7	1	1-4(3)	1-5(3)	1-4(2)	2-4(3)	2-5(3)	2-4(3)	1	-	-
8	1L	0	1	1	1	1	6,7(7)	8-10(8)	-	-
9	1	1	1	1	1	1	1	1,2(1)	1	-
10	4,5 (5)	-	-	1-2(1)L	1L	1 L	1L	1	-	-
11	3,4 (3)	-	1	1	1	1	1	1	-	-
12	1,2(1)	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	1	-	-

L- long; Wb- Weakly barbed; B- barbed, Bf- Bifid; F- fanlike setae, m- multiple (more than 10 branches)

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Table 6. Chaetotaxy of the larva of *Toxorhynchites darjeelingensis* sp. n.

Seta No	Head	Antenna	Thorax			Abdominal segments									
			Pro-(P)	Meso (M)	Meta (T)	I	II	III	IV	V	VI	VII	VIII	X	
0	-	-	M,d	-	-										
1	1	4,5(5)d	1	1	1 (L,B)	1 (L,B)	1 (B)	1 (B)	1 (B)	1 (B)	1 (B)	1 (L,B)	1,2(1)	1 (B)	
2	1	1	1,2(2)	1 (L)	2,3(2)	2	1	1	1	1	1	1	2,3(2)	8,9(8)	
3	1	1	5,6(6)	1	m,d	2 (B)	2(L,B)	1 (L,B)	1,2(1) (L,B)	1 (L,B)	1 (B)	1 (L,B)	3-5(3)	4-6(5)	
4	1 (L)	1	m, d	1	m,d	2(L,B)	2(L,B)	2(L,B)	1,2(1) (L,B)	1 (B)	5	2	1(L,B)	8,9 (8)(B)	
5	m,d	1	1 (B)	1 (L,B)	m,d	4-6(4)	4,5(5)	4,5(4)	4,5(4)	2(L,B)	2	1	1(L,B)	-	
6	1 (L)	1	4-6(4)	1 (Sf,B)	1 (Sf,B)	2 (B)	2(L,B)	2(L,B)	2(L,B)	2(L,B)	1(L,B)	m	-	-	
7	1 (L)	-	2 (Sf,B)	3,4(3)	2 (Sf,B)	2(L,B)	2(L,B)	2(L,B)	2(L,B)	1(L,B)	1(L,B)	1 (B)	-	-	
8	1 (L)	-	4,5(4) (L,B)	m	m,d	-	1	1	1	1	m	m	-	-	
9	4-6(4)(B)	-	1 (Sf,B)	1 (Sf,B)	1 (Sf,B)	2	2	2	1	1	1	1	-	-	
10	7-9(8)	-	1 (L,B)	1 (L,B)	1 (L,B)	1	1,2 (2)(L,B)	2(L,B)	2(L,B)	1 (B)	1 (L)	1	-	-	
11	5,6 (5)	-	1	1	2	2(L,B)	1,2 (2)(L,B)	2 (B)	2(L,B)	1(L,B)	1(L,B)	1 (L,B)	-	-	
12	4(B)	-	1 (L)	1 (L)	1 (L)	m,d	2,3(2)	3,4(4)	3,4(4)	3,4(4)	1	1	-	-	
13	-	-	-	2 (Sf, B)	1 (Sf,B)	1 (L,B)	1 (B)	1 (B)	1 (B)	1 (B)	1 (B)	1 (L,B)	-	-	
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

L-long seta; B-barbed; m- multiple (more than 10 branches); d- dendritic; Sf- Stiff. 1-S with 6,7(6)(Sf, B)

Table 7. Morphological characters of *Tx. darjeelingensis* sp. n. compared with *Tx. bengalensis*, *Tx. tyagii* and *Tx. splendens*

Species	<i>Tx. bengalensis</i>	<i>Tx. tyagii</i>	<i>Tx. splendens</i>	<i>Tx. darjeelingensis</i>
Mesonotum	No broad pale yellow scales over wing roots to scutellum	Broad pale yellow scales over wing roots to scutellum	No broad pale yellow scales over wing roots to scutellum	Broad pale yellow scales over wing roots to scutellum
Abdomen terga	Lateral tufts of VI-Te with long white and dark brown tufts; VII & VIII-Te with dark brown tufts	lateral tuft of VI-Te, 0.75 with black remaining yellow; VII-Te black; and VIII-Te orange	lateral tuft of VI-Te yellow and black; VII-Te black; and VIII-Te orange	lateral tuft of VI-Te 75% deep blue remaining black; VII-Te 90% deep-green remaining yellow; and VIII-Te yellow
Wing size	7.3mm	7.5mm	8.5mm	5.7mm
Legs	Mid and hind femora with metallic purple scales dorsal, and yellow scales ventral; tibiae with purple scales	Hind femur black; mid femur black with pale areas, fore femur with wide basal ring; all tibiae dark	Hind femur mainly pale golden, purple dorsally on distal ½, fore and mid femora purple, pale golden ventrally and posteriorly; fore tibia purple; mid tibia mainly pale golden, purplish at base and apex; hind tibia purple, with greenish reflections.	Hind femur 70% with golden yellow, remaining black, fore femur with wide basal ring, mid femur black with pale scales; fore and hind tibia dark, mid-tibia with white longitudinal strips
Male genitalia	Basal mesal lobe with 2 stout apical seta; gonostylus with few microsetae restricted to apical 1/3	Basal mesal lobe with 2 stout apical seta; gonostylus with few microsetae from base to apex	Basal mesal lobe with 1 stout apical seta; gonostylus with few microsetae restricted to distal half	Basal mesal lobe with 1 stout apical seta; gonostylus with numerous microsetae distributed evenly from base to apex.
Larva	Seta 7-M 4,5 branches, and 13-M multiple branches; seta 1-S with 10 branches	Seta 7,13-M with 5 branches; seta 1-S with 7 branches	Seta 7-P,M and 13-M double; seta 1-S with 7 branches	Seta 7-M with 3 branches and 13-M double, stiff with barbed; seta 1-S with 6 branches
Pupa	10-CT-12-CT with 2 branches; Seta 8- VII with 8 branches	10-CT with 11, 11-CT with single and 12-CT with 2 branches; Seta 8-VII with 10 branches	10-CT with 5, 11-CT with 2 and 12-CT with 4 branches; Seta 8-VII with 4 branches	10-CT with 5, 11-CT with 3 and 12-CT with single branches; Seta 6-VII with 8 branches

(vii) In *Tx. darjeelingensis* seta 1-VIII of abdominal segment arises from outside the large sclerotized plate and is without the basal tubercle. In the rest of species under discussion the 1-VIII originates from within the periphery of sclerotized plate and is embedded in a tubercular structure.

(viii) The molecular analysis of *Toxorhynchites* sp. n. (WB16) alludes its affinity with *Tx. splendens* rather than *Tx. rutilus*. The species analyzed are positioned in three separate branches in the phylogenetic tree. *Toxorhynchites rutilus* has branched much earlier (branch length 0.126). On the other hand, *Tx. darjeelingensis* sp.n. has branched out (0.035) much lately but slightly before *Tx. splendens* and *Tx. tyagii* clustered branch (0.032).

These differences in various morphological structures as well as the branching time of different *Toxorhynchites* species clearly indicates that each species has evolved separately at different points of time; thus confirming *Tx. darjeelingensis* to be clearly a distinct and hitherto undescribed species.

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