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Morphology and molecules say: *Tanytarsus latens*, sp. nov. from Finland (Diptera: Chironomidae)

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Abstract

Tanytarsus latens **sp. nov.** is described from Finland (Ostrobothnia borealis, Satakunta). Both morphological and molecular analyses indicate that *T. latens* belongs to the *mendax* species group. The adult male hypopygium of the new species resembles that of *Tanytarsus occultus* Brundin and of *T. desertor* Giłka *et* Paasivirta, while the molecular analysis based on the mitochondrial cytochrome oxidase (COI) gene fragment evidences that *T. latens* is a sister species to most of European *Tanytarsus* of the *mendax* group's core, for which the COI barcodes are known. Notes on biology of *T. latens* are also provided.

Key words: Diptera, Chironomidae, systematics, DNA barcoding, new species, Finland

Introduction

Chironomidae, with nearly 7,500 specific and 550 generic names, is one of the largest and most diverse dipteran families (Pape *et al.* 2011). The species richness in the 12 known chironomid subfamilies is, however, much unequal, and the majority of described species belongs to Orthocladiinae and Chironominae. Tanytarsini is one of three tribes in the subfamily Chironominae. It comprises approximately 10% of all known chironomid species, including those belonging to the largest genus *Tanytarsus* van der Wulp. Recent inventories indicate that *Tanytarsus* comprises 355 known valid species worldwide (Lin *et al.* 2018b). Most of them are clustered into species groups. Systematic relationships within and between several species-rich groups, in particular the *chinyensis*-, *eminulus*-, *gregarius*-, *lugens*- and *mendax* groups, have been comprehensively analysed on the basis of both morphology and DNA sequences, including the best-known species recorded from Europe (e.g. Ekrem 2001, 2002, 2003, 2004; Ekrem *et al.* 1999, 2003; Giłka & Paasivirta 2007; Giłka 2010, 2011a, b; Lin *et al.* 2017, 2018b). However, the above groups have recently been profoundly revised, and the previously proposed concepts were refuted (Lin *et al.* 2017, 2018b). As a result, both the *chinyensis*- and *eminulus* groups were split into several groups, the *gregarius*- and *lugens* groups were merged, while a core of the *mendax* group was redefined after exclusion of *Tanytarsus aculeatus* Brundin, *T. formosanus* Kieffer and *T. ovatus* Johannsen.

Major pros of molecular methods, such as DNA barcoding, in complementing the morphological studies are evident in the case of immature stages, damaged and neglected specimens, when phenotypic characters are difficult to define or if a researcher lacks experience to identify taxa on the basis of their morphology (Ball *et al.* 2005, Geraci *et al.* 2011, Hebert *et al.* 2003, Janzen *et al.* 2005, Savolainen *et al.* 2005, Montagna *et al.* 2016a). DNA-based diagnostic characters may also serve as the backbone for a taxonomic description of newly discovered cryptic species (e.g. Anderson *et al.* 2013).

The DNA barcoding or, more recently developed, metabarcoding have been proposed, and successfully adopted as an efficient method for species identification and habitat biodiversity assessment using standardized

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genetic markers (Andrews *et al.* 2016, Arribas *et al.* 2016, Carew *et al.* 2013, Wang & Wang 2012, Yu *et al.* 2012). Capability of these methods has been enhanced by the development of standardized DNA-barcode reference databases (e.g. BOLD, GENBANK and EMBL). However, it needs to be underlined that reliability of the taxonomic assignment of deposited reference sequences may vary due to the still poorly developed quality-check procedures, and must be treated with caution. On the other side, relevant advantages of using short DNA fragments to characterize the organisms' diversity rely on the short time and low price required to pursue the proposed goals (Meier *et al.* 2015). Thus, such methods provide a handy aim in rapid and cost-efficient discovery of new species in the times of taxonomic impediment (e.g. Ebach *et al.* 2011).

Our present study is focused on a new species found in Finland, the country considered to be well investigated, though still revealing chironomid species new to science (e.g. Giłka 2005; Giłka & Paasivirta 2007, 2008, 2009; Puchalski *et al.* 2018). The adult male of the new species shows a distinct morphological similarity to those of *Tanytarsus desertor* Giłka *et* Paasivirta and *T. occultus* Brundin, and is identified as a member of the *mendax* group. Following the recent systematic concept (Lin *et al.* 2018b), it is the seventh species of this group recorded from Europe, and the sixth species known from Fennoscandia.

Material and methods

Sampling, processing and deposition of the type material. The examined individuals were collected using a hand net and Malaise traps, and slide-mounted in Canada balsam or Euparal. Thoraces of the holotype and one paratype specimen, preserved in ethanol, were used for DNA extraction. Measurements are in µm (except for data on the wing length given in Table 2); the lengths of leg segments and palpomeres have been rounded off to the nearest 5 and 1 µm respectively; the antennal, leg and venarum ratios (AR, LR, VR) have been calculated to the second decimal place. The morphological terminology and abbreviations follow Sæther (1980). The photographs were taken using a Leica DM6000 microscope and LAS Montage multifocus. The type specimens are housed in the Laboratory of Systematic Zoology, Department of Invertebrate Zoology and Parasitology (DIZP), University of Gdańsk, Poland, and in the personal collection of L. Paasivirta, Salo, Finland (LP). The DNA voucher (extracted DNA in elution buffer) is deposited in the Museum and Institute of Zoology Polish Academy of Sciences (MIZ PAS), Warsaw, Poland. Catalogue number: MIZ-TANY002-18; Barcode Index Numbers Algorithm (BIN): BOLD:ADM4007 Sample ID: IM_To-01, IM_To-02, Process ID: TANY001-18, TANY002-18.

Molecular analysis. Fragments of 96% EtOH-fixed thoracic muscle tissue from two individuals of the new species, stored at 4ºC, were used for the total DNA extraction with the GenoPlast Tissue Genomic Extraction Mini Kit (GenoPlast Biochemicals), following the manufacturer protocol. Prior to the extraction, *ca.* 2 mm^3 of the muscle tissue was incubated overnight at 60ºC in a 1.5-ml tube containing 200µl of GATG2 lysis buffer with 20µl of proteinase K (10 mg/ml) (Seutin *et al.* 1991). Finally, the extracted DNA was eluted in 100µl of elution buffer, pH 8.00, stored at 4ºC until amplification and finally long-term stored at -20ºC.

A standard *ca.* 600 bp long mitochondrial COI barcode marker was PCR-amplified from each specimen, using the universal LCO1490 and HCO2189 primer pairs (Folmer *et al.* 1994), that had been successfully employed in previous studies upon Chironomidae (Anderson *et al.* 2013; Lin *et al.* 2015, 2017, 2018b; Montagna *et al.* 2016b; Song *et al.* 2018). Each amplification was conducted in a volume of 25µl, including DreamTaq Master Mix (1x), Polymerase (Thermo Scientific Inc.), 400nM of each primer and 2 µl DNA template on a BIO-RAD T100 Thermal Cycler.

The two-step PCR settings included initial denaturing at 94ºC for 60s, five cycles of denaturation at 94ºC for 30s, annealing at 45ºC for 90s, extension at 72ºC for 60s, followed by 35 cycles of denaturation at 94ºC for 30s, annealing at 51ºC for 90s, extension at 72ºC for 60s, and final extension at 72ºC for 300s (Hou *et al.* 2007). A 2 µl aliquot of each PCR product was visualized in MidoriGreen-stained (Nippon Genetics) 1.0% agarose gel to check for the product quality and length. Then, the PCR products were purified with exonuclease I (20 U mL-1, Fermentas) and alkaline phosphatase FastAP (1 U/mL, Fermentas) according to the manufacturer's guidelines. Subsequently, the products have been sequenced using the same primers as at the amplification stage. Sequencing of the PCR products was performed using BigDye terminator technology by Macrogen Inc. Netherlands.

Both resulting sequences were verified and confirmed as belonging to *Tanytarsus* via BLAST (GenBank) and BOLD identification engines. The sequences were aligned with fourteen COI sequences of other *Tanytarsus*

species that were fetched from BOLD using MAFFT plugin in Geneious software (Katoh & Standley 2013). The resulting alignment was finally trimmed to the length of 528 bp. The two sequences obtained from the new species represented one haplotype. Both were uploaded to BOLD, in order to obtain the Barcode Index Number (BIN) being an operational species equivalent, and to GenBank (Specimen Accession Numbers: TANY001-18, TANY002-18; Sequence Accession Numbers: MH592977, MH592978). Additionally, the images of hypopygium of the type individuals as well as other information on the voucher specimens were uploaded to BOLD.

The phylogenetic relationships between the newly described species and other studied members of the *Tanytarsus mendax* group were illustrated with a Neighbor-Joining (NJ) phylogram (Saitou & Nei 1987) with bootstrap test performed on 1000 replicates (Felsenstein 1985), based on our data and the 14 other COI sequences mined from BOLD. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980). The NJ phylogeny was also validated with the Maximum Likelihood method, under the GTR+G model and bootstrap test with 1000 replicates (Nei & Kumar 2000). The sequences of *Tanytarsus gracilentus* (Holmgren) from the *norvegicus* group were tested as an outgroup. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The Kimura 2-parameter distances averaging over all sequence pairs within and between each group were also calculated. All the analyses were conducted in MEGA7 (Kumar *et al.* 2016).

Results

Systematics

Family: Chironomidae Newman, 1834
Subfamily: Chironominae Newman, 1834
Tribe: Tanytarsini Zavřel, 1917
Genus: *Tanytarsus* van der Wulp, 1874

Tanytarsus latens sp. nov.

Type material. Holotype (DIZUG), adult male specimen (excl. thorax) slide-mounted in Canada balsam: FINLAND, OSTROBOTHNIA BOREALIS, Palokkaanlampi and Rumajärvi *ca.* 40 km W of Rovaniemi (66°26'N 24°56'E), 7–28 August 2017, Malaise trap, J. Salmela. Paratypes: sampling data as holotype: 1 male (excl. thorax) in Canada balsam, 5 males + 1 hypopygium in Euparal (DIZUG); SATAKUNTA, Kauklastenjärvi *ca.* 15 km SE of Rauma (61°04'N 21°46'E), 17 August 2010, 1 male (in Euparal), hand net, L. Paasivirta (LP). DNA voucher: DNA extracted from thorax tissue in elution buffer (MIZ PAS).

Derivatio nominis. From the Latin adjective meaning concealed or disguised by others.

Diagnosis. Darkly coloured, relatively big [wing length: 2245–2540 (2430) µm]. Frontal tubercles small, 2–12 µm long at most. Anal tergite bands of H-type, with broad median connection. Anal point slender, with narrowly rounded apex and 2–3 spinulae. Superior volsella rounded at base, elongate, tapering towards tip, usually with apical nose curved medially. Stem of median volsella straight, slightly swollen in distal half, with irregularly arranged subulate lamellae.

Description. **Adult male** (n = 8 + 1 male hypopygium).

Colouration (in alcohol). Eyes black. Antenna, tentorium, scutal stripes, scutellum, postnotum, sternum, hypopygial apodemes and proximal leg segments, incl. femora and tibiae dark brown to black. Head capsule, mouthparts, ground colour of thorax, tarsi and abdomen brown with slight olive undertone. Wing and haltere pale brownish.

Head. Eyes reniform, with dorsomedian extension gradually narrowing from 5 facets at base to 4: 4: 3/2 facets medially. Antenna with 13 distinct flagellomeres, AR 1.53–1.78 (1.66), plume fully-developed. Frontal tubercles minute, usually in shape of tiny swellings (2–3 µm), rarely conical or cylindrical, 12 µm long at most (Fig. 1A, B). Lengths of palpomeres 2–5 (in µm): 60–72 (63), 155–183 (172), 143–163 (156), 198–258 (244); pm_3 always longer than pm_4. Clypeus with 17–24 setae.

Thorax chaetotaxy (n = 5). Ac 20–22, biserial, with several setae arranged in small field near antepronotum; Dc 11–14 on each side; Pa 1–2 on each side; Scts 4–8, usually 8.

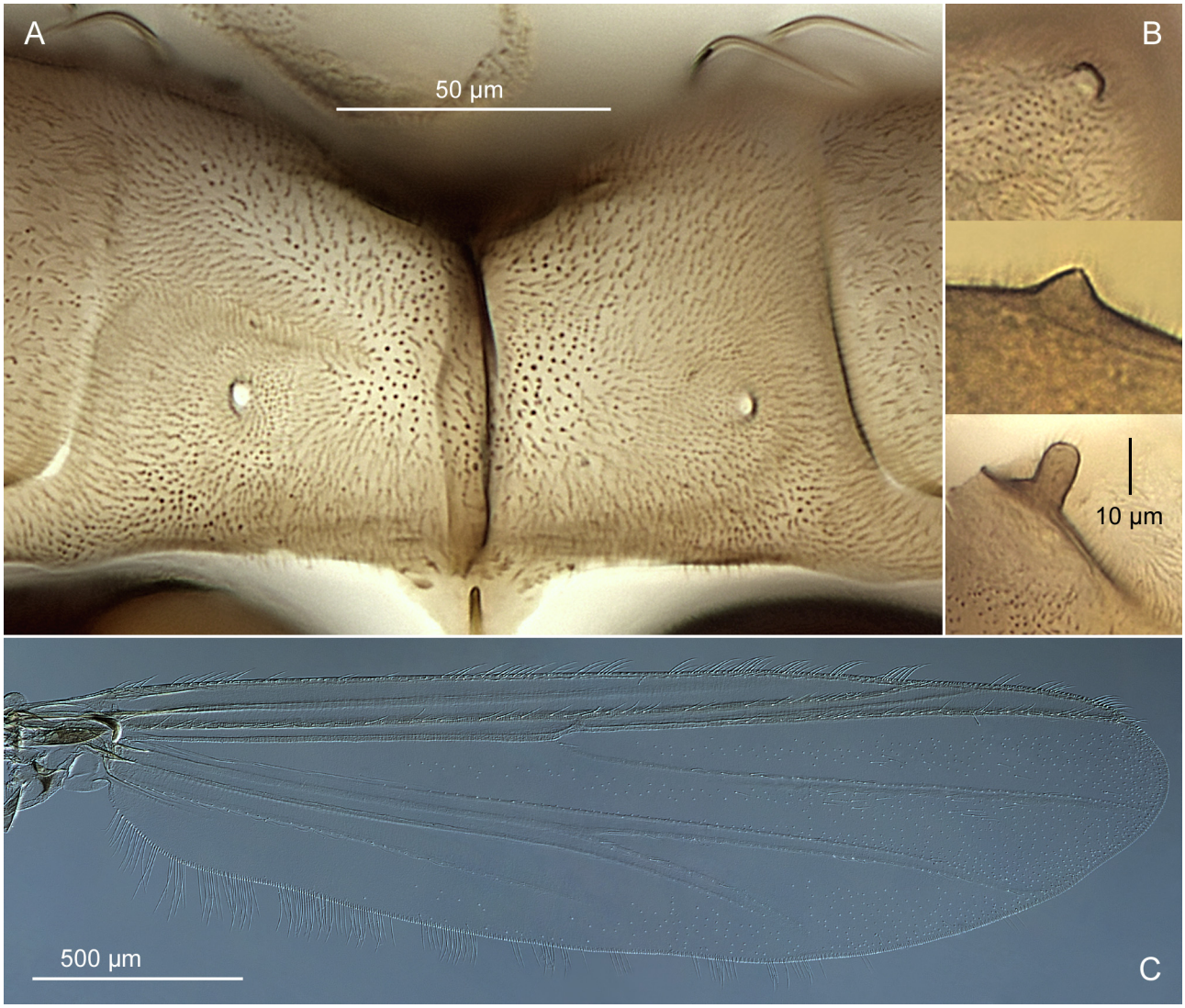

FIGURE 1. *Tanytarsus latens* **sp. nov.**, male. Frons (**A**) and frontal tubercles (**B**), wing (**C**).

Wing (Fig. 1C). Length 2245–2540 (2430) µm. Venation pattern and chaetotaxy typical of the genus, as shown in Fig 1C; VR_{Cu} 1.14–1.16 (n = 2).

Legs. Fore leg tibia with slightly curved distally spur 25–30 µm long. Mid and hind leg tibiae with combs separated, each comb bearing spur; spurs straight or slightly curved, 28–36 µm long on mid leg and 40–55 µm long on hind leg. Basitarsus of mid leg with 4–7 sensilla chaetica. Lengths of leg segments and leg ratios as shown in Table 1.

TABLE 1. Leg segment lengths (µm) and leg ratios of male *Tanytarsus latens* **sp. nov.**

	fe	ti	ta_1	ta_2	ta_3	ta_4	ta_5	LR
p_1	895–1030 (990)	610–715 (685)	1050–1225 (1180)	575–685 (640)	480–565 (535)	355–435 (405)	165–205 (190)	1.71–1.75 (1.73)
p_2	935–1065 (1025)	840–955 (930)	485–580 (545)	295–355 (330)	230–270 (255)	160–195 (185)	110–140 (130)	0.58–0.61 (0.59)
p_3	1080–1235 (1190)	1115–1300 (1260)	720–865 (820)	455–535 (510)	375–450 (420)	250–310 (280)	140–170 (160)	0.64–0.67 (0.66)

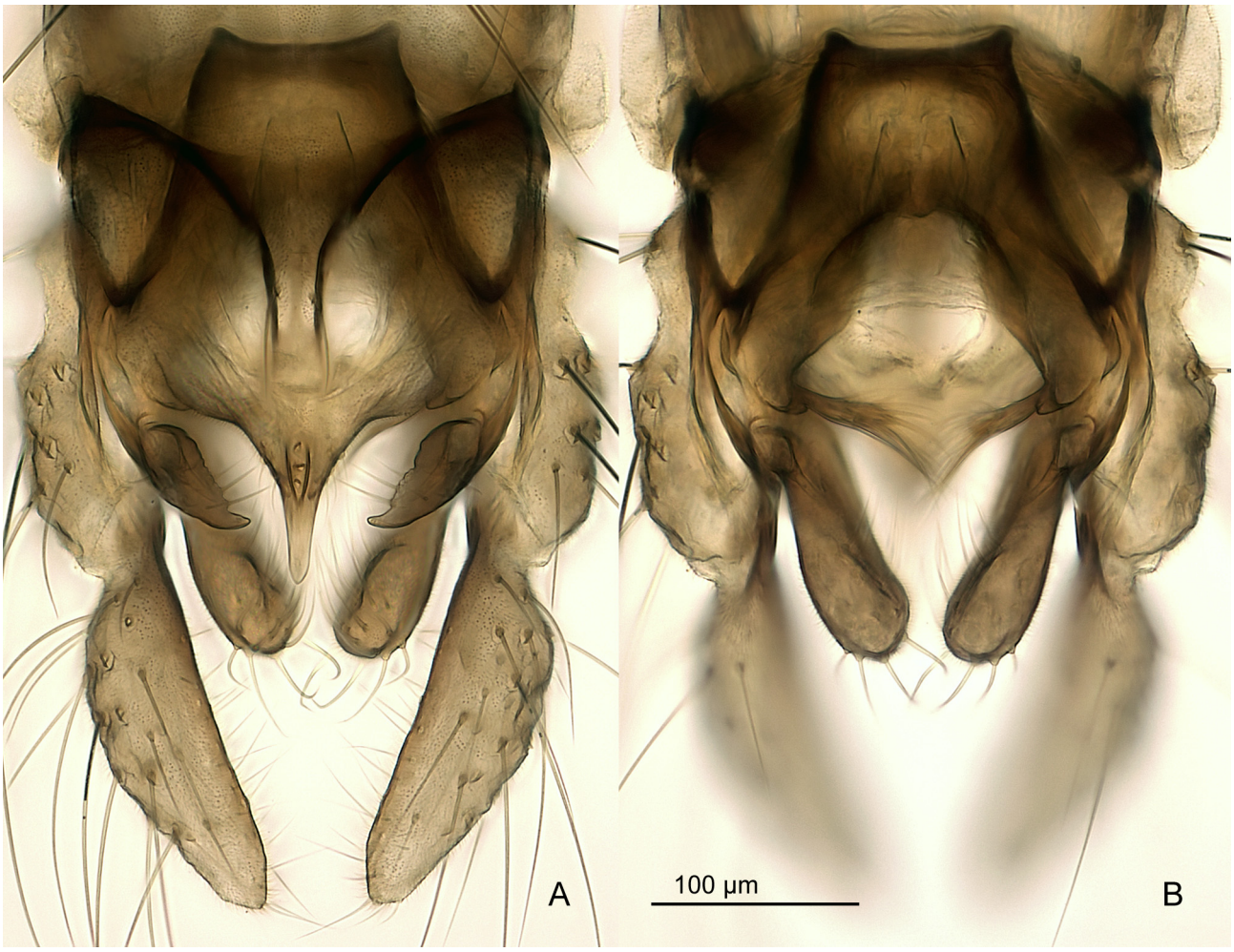

FIGURE 2. *Tanytarsus latens* **sp. nov.**, male hypopygium in dorsal (**A**) and ventral view (**B**).

Hypopygium (Figs 2, 3). Gonostylus 160–180 µm, longer than gonocoxite. Anal tergite bands of H-type, with broad median connection (Fig. 2A). Anal tergite usually with 2 long median setae, rarely 3 setae present (n = 2) or median setae absent (n = 1); extensive microtrichia-free area surrounding posterior sections of anal tergite bands; lateral teeth and lateral setae absent; shoulders on posterior margin weak (Figs 2A, 3A). Anal point slender, evenly tapering towards narrowly rounded apex, bearing 2–3 well-developed spinulae placed between crests (Figs 2A, 3A). Superior volsella rounded at base, elongate, tapering towards narrow tip, usually with apical nose curved medially, 4–6 dorsal setae and 3 setae on anteromedian margin (proximal seta weaker), microtrichia absent; digitus long, but not extending beyond margin of superior volsella, pointed (Figs 2, 3B). Stem of median volsella 40–45 µm long, straight, slightly swollen in distal half, bearing several setiform and subulate, irregularly arranged lamellae (Fig 2B). Inferior volsella straight, stout, with distal part swollen, roundish, bearing numerous strong setae (Figs 2A, B).

Morphospecies of the *mendax* group and their DNA barcodes

According to the recent concept (Lin *et al.* 2018b), *Tanytarsus latens* is the seventh species of the *mendax* group recorded from Europe, along with *T. desertor* Giłka *et* Paasivirta, *T. mancospinosus* Ekrem *et* Reiss, *T. mendax* Kieffer, *T. occultus* Brundin, *T. volgensis* Miseiko, and *T. tika* (Tourenq). Diagnostic morphological characters found in the adult males of *T. latens* allow us to identify them as closely related to those of *T. desertor* and *T. occultus*. The detailed character analysis, however, indicated several differences between these species, as shown in Table 2.

TABLE 2. Morphological diagnostic characters for male *Tanytarsus desertor* Giłka *et* Paasivirta, *T. latens* **sp. nov.**, and *T. occultus* Brundin. * - specimens sampled together with *T. latens*.

character/species	*T. desertor*	*T. latens*	*T. occultus* *
Colouration	light green to light brown	olive brown to dark brown/black	yellowish-green to brown
Size, wing length	~1.55 mm	~2.25–2.55 mm	~2.00–2.05 mm
Frontal tubercles	well-developed, 15–20 µm	small, 2–12 µm	stout, 30 µm
Gonostylus length	120–130 µm	160–180 µm	130–140 µm
Anal tergite bands	V-type, separated	H-type, broadly connected	V-type, separated
Median setae	absent	present	present
Microtrichia-free area of anal tergite	small	extensive	extensive
Anal point apex	blunt, transversely cut or slightly concave	narrowly rounded	square or distinctly concave
SVo	broadened apically, with ventromedian lip	tapering towards apex, usually with distinct apical nose	tapering towards apex, with apical protuberance at most
MVo lamellae	apically blunt, fan-folded	pointed, irregularly arranged	pointed, irregularly arranged

Most recent taxonomic studies describing new species are providing DNA barcodes as a new character in description of new species and species delimitation of chironomids (Cheng & Wang 2006, Krosch *et al.* 2011, Lin *et al.* 2017, Yan *et al.* 2017, Song *et al.* 2018). When a new species is described, the molecular data provide a useful insight in the scale and thresholds of intra- and interspecific distances that may help with the decision on the taxonomic status. The topology of the Neighbor-Joining/Maximum Likelihood phylogram shows that the newly described *Tanytarsus latens* is in sister relationships to the clade consisting of *T. volgensis*, *T. desertor* and *T. occultus*, while *T. mendax* is sister to all these species (Fig. 4).

The threshold of 0.04–0.05 K2P distance was first reported as an appropriate level for species discrimination within Chironomidae (Lin *et al.* 2015, Meier *et al.* 2015). On the other side, Carew and Hoffmann (2015) used 0.07 K2P as a threshold for closely related species in Chironomidae. Most recently, Lin *et al.* (2017, 2018a) proposed even more conservative approach with up to 0.08 intraspecific divergence in the *Tanytarsus heusdensis* species complex and 0.10 in the *T. curticornis* complex. In the presently analysed species of the *mendax* group, the minimum intraspecific genetic distance is 0.011 in *T. mendax* and maximum is 0.058 in *T. occultus* (Table 3).

TABLE 3. Estimates of average K2P distance over sequence pairs within the studied species of the *mendax* group. * - not applicable (single sequence or sequences obtained from specimens representing one haplotype).

Species	K2P Distance
Tanytarsus mendax	0.011
Tanytarsus volgensis	0.011
Tanytarsus occultus	0.058
Tanytarsus latens	*
Tanytarsus desertor	*

Within the *mendax* group, the interspecific genetic distance between the newly described *T. latens* and the other species starts from *ca.* 0.14 K2P, while the distance between other species is from over 0.133 between *T. desertor* and *T. occultus*, up to 0.220 between *T. latens* and *T. volgensis* (Table 4). As such, the observed distances fulfil even the most conservative distance threshold for species discrimination, and support the idea of the newly described *T. latens* as a distinct species. It is worth to note that *Tanytarsus gracilentus* of the *norvegicus* group, here treated as an outgroup, displays a rather low interspecific genetic distance in relation to the examined species of the *mendax* group's core. The interspecific distance between *T. gracilentus* and the species from the *mendax* group ranges from 0.157 (*T. gracilentus* vs. *T. mendax*) to 0.234 (*T. gracilentus* vs. *T. volgensis*) (Table 4). This may

support the concept of close relationships between the two groups, as proposed by Lin *et al.* (2018b), and explains hesitations in defining the group membership of *T. gracilentus* - previously suggested as a possible member of the *lugens*- or the *mendax* species group (Ekrem *et. al.* 2003).

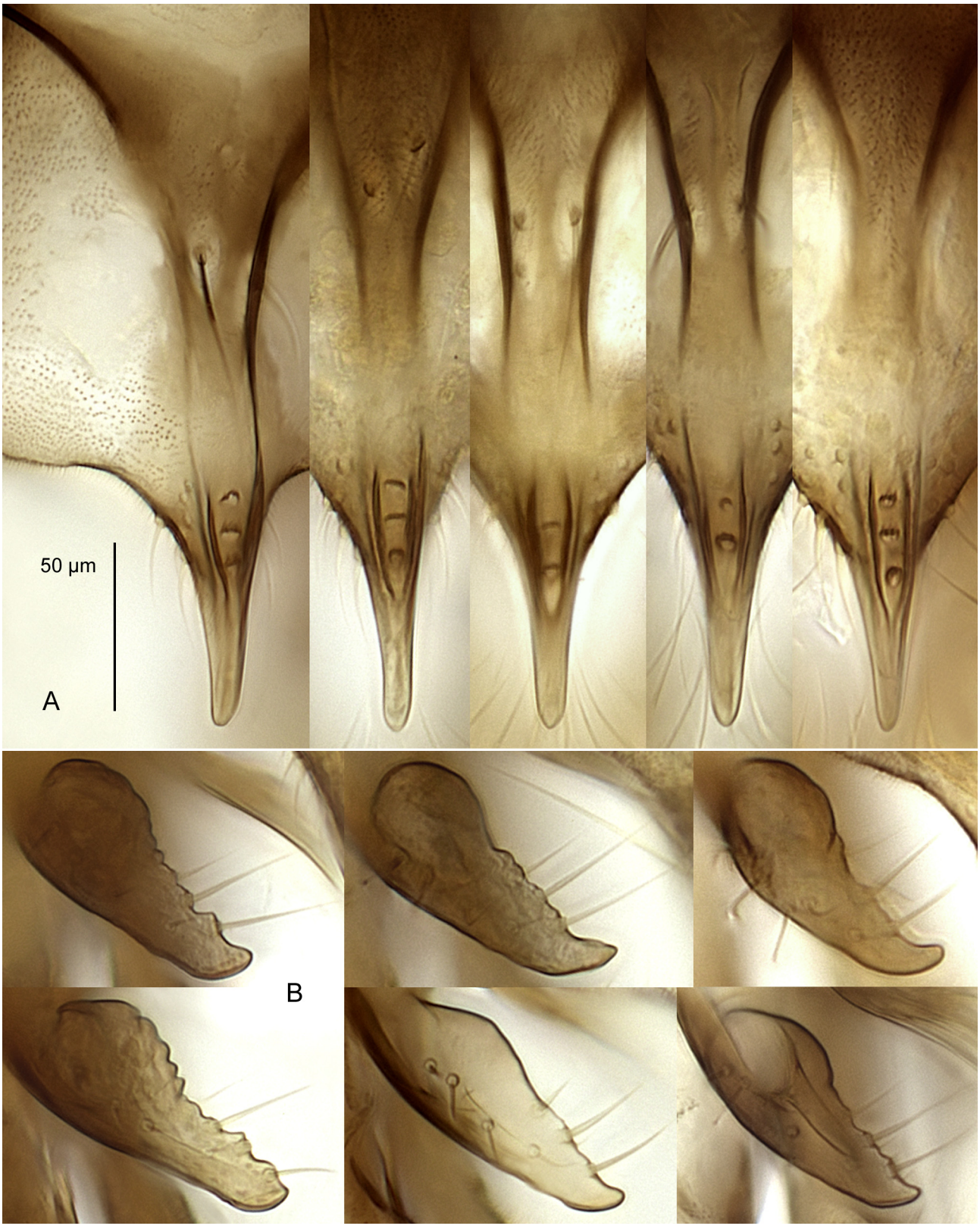

FIGURE 3. *Tanytarsus latens* **sp. nov.**, variations in hypopygial anal point (**A**), and superior volsella and digitus (**B**).

TABLE 4. Estimates of average K2P distance over sequence pairs between the studied species of the *mendax* group's core and *T. gracilentus* (outgroup).

Species	K2P Distance				
Tanytarsus latens					
Tanytarsus desertor	0.144				
Tanytarsus occultus	0.147	0.133			
Tanytarsus volgensis	0.220	0.155	0.182		
Tanytarsus mendax	0.159	0.151	0.155	0.186	
Tanytarsus gracilentus	0.187	0.194	0.180	0.234	0.157

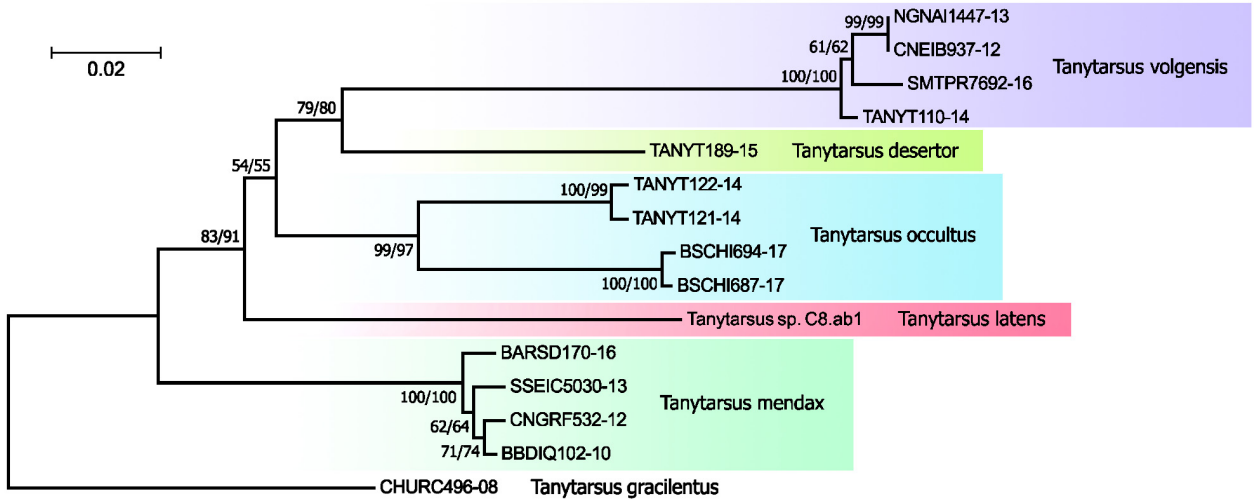

FIGURE 4. Phylogram showing evolutionary relationships within five species of the *Tanytarsus mendax* species group's core, based on the 528 bp long COI gene fragment. Numbers by nodes indicate bootstrap values (only values >52% are shown). Number values by nodes indicate bootstrap values from Neighbor-Joining/Maximum Likelihood trees.

Biology. The records of *Tanytarsus latens* come from two distant localities. In northern Finland (Ostrobothnia borealis), the adult males were collected at meso-eutrophic fens and permanent shallow boggy ponds surrounding two small lakes with marshy shores: Palokkaanlampi (Fig. 5A) and Rumajärvi (Fig. 5B) situated *ca.* 600 m from each other. A single male was also sampled at the highly eutrophic shallow lake Kauklastenjärvi in southern Finland (Satakunta). These data allow us to presume that the larvae of *T. latens* dwell in nutrient-rich bog ponds or marshy shores of lakes. A list of species occurring together with *T. latens* confirms that the new species inhabits this kind of lacustrine habitats. *T. latens* was taken together with *Tanytarsus aberrans* Lindeberg, *T. curticornis* Kieffer, *T. gregarius* Kieffer, *T. inaequalis* Goetghebuer, *T. lestagei* Goetghebuer and *T. verralli* Goetghebuer on the sites in northern Finland, with *T. nemorosus* Edwards on the south, and with *T. occultus* Brundin, *T. striatulus* Lindeberg and *T. telmaticus* Lindeberg on all the sites explored. *T. latens* seems to be a univoltine species emerging only in the late summer (August).

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Data availability. The following information was supplied regarding data availability: GenBank and BOLD databases. The DNA sequences are deposited in the BOLD dataset, which is publicly available, (BIN): BOLD: ADM4007.

FIGURE 5. Sampling sites (type locality) of *Tanytarsus latens* **sp. nov.** in northern Finland (Ostrobothnia borealis): Palokkaanlampi (**A**) and Rumajärvi (**B**). Photos by Jukka Salmela.

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