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Molecular phylogeny of Acerentomidae (Protura), with description of *Acerentuloides bernardi* sp. nov. from North America

Julia Shrubovych[1,2,], Josef Starý[3], and Cyrille A. D'Haese[4]*

Abstract

The closely related genera *Acerentulus* Berlese, 1908 and *Acerentuloides* Ewing, 1921 are represented in the USA by only 3 species: *Acerentulus confinis* (Berlese, 1908), *Acerentulus christensoni* (Ewing, 1940), and *Acerentuloides americanus* (Ewing, 1924). Study of proturan material from North America yielded a new species belonging to *Acerentuloides*. The new species is characterized by the absence of seta sd4 on the head, presence of a minute apical median seta on abdominal legs II and III, unique shape of maxillary gland with distal row of clustered globules, presence of setae modified as short, thickened sensilla (sd5 on head, β1 and δ4 on foretarsus, setae P4 on metanotum and A2 on thoracic sterna, and accessory setae on tergites and sternites I–VI), absence of seta P1a on abdominal tergites I–VI, foretarsal sensillum b longer than c, and presence of psl pores only on tergite VI. Molecular sequences for several Acerentomidae, including *A. confinis*, were used to develop hypotheses about species relationships within Acerentomidae.

Key Words: *Acerentulus*; *Podolinella*; Indiana; USA; DNA barcoding; phylogeny

Resumen

Los géneros *Acerentulus* Berlese, 1908 y *Acerentuloides* Ewing, 1921, muy relacionados entre sí, están representados en Estados Unidos por sólo 3 especies: *Acerentulus confinis* (Berlese, 1908), *Acerentulus christensoni* (Ewing, 1940) y *Acerentuloides americanus* (Ewing, 1924). El estudio del material de proturas de Norteamérica resulto en una nueva especie perteneciente a *Acerentuloides.* La nueva especie se caracteriza por la ausencia de seta sd4 en la cabeza, la presencia de una seta muy corta en el apice central de las patas abdominales II y III, la forma única de la glándula maxilar con una hilera distal de glóbulos agrupados, la presencia de setas modificadas como sensilas cortas y gruesas (sd5 en cabeza, β1 y δ4 en tarso anterior, setae P4 en el metanotu y A2 en esternón torácico y setas accesorias en tergitos y esternitos I-VI), ausencia de seta P1a en tergitos abdominales I-VI, sensillum b de primer tarso más largo que c, y la presencia de poros psl solamente en el tergito VI. Se utilizaron secuencias moleculares para varios Acerentomidae, incluyendo *A. confinis*, para desarrollar la hipótesis sobre las relaciones de especies dentro de Acerentomidae.

Palabras Clave: *Acerentulus*; *Podolinella*; Indiana; Estados unidos; códigos de barras de ADN; filogenia

The history of the work on the genus *Acerentuloides* (Entognatha: Protura: Acerentomidae), especially of its type species, *Acerentuloides americanus* (Ewing, 1924), is worth examining. The genus was originally described by Ewing (1921) with *Acerentuloides bicolor* Ewing, 1921 as its type species. The species *A. americanus* was originally described by Ewing (1924) as *Acerentomon americanum* (Ewing, 1924). *Acerentomon americanum* was transferred to the genus *Acerentulus* after revision of Ewing's type material of Protura (Hilton 1943; Bonet & Tuxen 1960). Tuxen (1963) created for this species the new genus *Amerentulus* Tuxen, 1963 with the type species *Amerentulus americanus* (Ewing, 1924), despite the fact that type material was lost and Tuxen had doubts about the validity of the new genus (Tuxen 1964, p. 225). Nosek & McEwan Kevan (1984, p. 59), studying Canadian specimens of *A. americanus*, re-established the generic name of *Acerentuloides* and synonymized it with *Amerentulus*, with the type species *Acerentuloides americanus*.

The genus *Acerentuloides* contains only the single species *A. americanus*, distributed in the USA and Canada (Szeptycki 2007). The morphologically similar genus *Acerentulus* comprises 49 species (Szeptycki 2007; Wu & Yin 2007; Shrubovych et al. 2012, 2014b; Galli & Capurro 2013) and is distributed worldwide, but in North America only 2 species are known: *Acerentulus confinis* (Berlese, 1908) and *Acerentulus christensoni* (Ewing, 1940). In the present paper, we describe a second species of *Acerentuloides*, provide a generic diagnosis, and discuss the phylogenetic position of the genus *Acerentuloides*, subfamily Berberentulinae and family Acerentomidae within Acerentomata.

Materials and Methods

Material of the new species was collected in the framework of a soil biota study in post-mining sites in the USA (Frouz et al. 2013). Samples were collected from litter and rhizosphere of deciduous forest with dominant black locust tree (*Robinia pseudoacacia* L.; Fabaceae) and red oak (*Quercus rubra* L.; Fagaceae). The forest patch is a 16-yr-old

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re-growth after coal mining in the Patoka River National Wildlife Refuge and Management Area near the Hugh Boyd Fishing Pier & Wildlife Observation Deck, near Oakland City, Gibson County, Indiana. Specimens of *A. confinis* were collected from leaf litter at the base of white oak in woods in Calhoun, McMinn County, Tennessee (35.295833°N, 84.748056°W), on 6 Aug 2013 by E. C. Bernard and K. J. Whitlock. The specimens were extracted from soil and moss samples with a Berlese–Tullgren funnel into 95% ethanol. Specimens were mounted on microscope slides in Marc André II medium (Massoud 1967) and were examined with a compound microscope with differential interference contrast optics at magnifications ranging from 250× to 1000×. Drawings were made with a drawing tube. Specimens for scanning electron microscopy (SEM) observations were preserved in 100% ethanol before critical point drying (Emitech K850, Quorum Technologies Ltd, Laughton, England) and sputter gold coating, and imaged with a Jeol JFC-1200 (JEOL Ltd, Tokyo, Japan) scanning electron microscope.

All synonymies, systematics, and literature data up to 2006 are given in Szeptycki (2007). Head seta designations follow Rusek et al. (2012); terminology for body chaetotaxy and porotaxy follows Szeptycki (1988, 1993) and Shrubovych (2014), body indices (PR, CF, BS, TR, EU) follow Tuxen (1964). Abbreviations used in the description are as follows: Abd = abdominal segments, al = anterolateral pore, cp = clypeal pore, fp = frontal pore, psl = posterosublateral pore, psm = posterosubmedial pore, spm = sternal posteromedial pore, spsm = sternal posterosubmedial pore, Th = thoracic segments.

In addition to the morphological description, the DNA barcode sequences were amplified to enhance the description (Stevens et al. 2011). DNA was extracted from 95% ethanol preserved specimens with a Qiagen DNeasy tissue extraction kit (Qiagen, Hilden, Germany). The specimens were digested overnight in buffer + proteinase k solution, and the extraction was carried out according to the manufacturer's recommendations with final re-suspension in 125 µL of the elution buffer. The voucher specimens were then retrieved, further cleared in lactic acid solution for approximately 24 h, and mounted on microscope slides in Marc André II mounting medium. Polymerase chain reaction (PCR) for DNA amplification was carried out in a 25µL volume reaction with Taq-&LOAD™ Mastermix 5xC reagent (MP Biomedicals, Santa Ana, California). The thermocycler program consisted of an initial denaturing step at 94 °C for 2 min, 5 amplification cycles with a 45 °C annealing temperature (94 °C for 40 s, 45 °C for 40 s, 72 °C for 1 min), 30 cycles with a 51 °C annealing temperature, and a final extension step at 72 °C for 5 min.

The PCR amplification and sequencing were carried out with primers newly designed for Protura: LCOprot (5'-TTT CTA CTA ATC ATA AGG ATA TTG G-3') and HCOprot (5'-TAN ACY TCW GGR TGS CCA AAR AAT CA-3'). We also selected 3 other loci to amplify for the phylogenetic analyses: *18S rRNA* and *28S rDNA* (regions D1–D2 and D3–D6) nuclear ribosomal DNA. The *18S rRNA* loci were PCR amplified in 3 overlapping fragments using primer pairs 1F + 5R, 3F + 18Sbi, and 5F + 9R (e.g., Giribet et al. 2004). For the *28S rDNA* regions, details are described in Schneider & D'Haese (2013) and Stevens & D'Haese (2014), and several primer pairs were used combining Collembola and Protura specific primers (some newly designed for the present work): 28S-C1c (5'-ACC CGC YWA ATT TAA GCA T-3') and 28S-ApR (5'-CGT GTT TCA AGA CGG GYC-3') for *28S rDNA* D1–D2; and 28S-Ap (5'-GRC CCG TCT TGA AAC ACG-3') and 28S-D4D5R (5'-GTT ACA CAC TCC TTA GCG GA-3') for *28S rDNA* D3–D6.

The PCR products were sequenced by Eurofins (Paris, France). Resulting chromatograms were interpreted using the program Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, Michigan) or CodonCode Aligner 6.0.2 (CodonCode Corporation, Centerville, Massachusetts). Sequences were manually checked for misreadings of the chromatograms.

Phylogenetic affinities were analyzed by comparing the obtained *COI*, *18S rRNA*, *28S rDNA* D1–D2, and *28S rDNA* D3–D6 sequences with sequences of other members of Acerentomidae published in previous works (Shrubovych et al. 2012, 2014b,c, 2016; Resch et al. 2014) completed with published sequences (Bu & Wu 2012; Bai & Bu 2013) and other unpublished sequences found in BOLD (Table 1). Other Acerentomata genera (*Neocondeellum* [Protentomidae], *Proturentomon* [Protentomidae], and *Hesperentomon* [Hesperentomidae]) were used to root the trees (see Table 1). Some of the *COI* sequences downloaded from GenBank or the Barcode of Life Data System (BOLD) were shorter than the barcode 658 bp so the missing ends of the alignment were completed with Ns. Sequences were aligned, for each locus independently, using MUSCLE v3.8.31 with default parameters (Edgar 2004).

Maximum likelihood (ML) analyses were performed using the partitioned ML method in RaxML (Stamatakis 2014) through the graphical interface raxmlGUI v1.5 (Silvestro & Michalak 2012). A thorough ML tree search was conducted with the GTR + Γ+ I model through 10 runs, and nodal branch support was evaluated with 1,000 bootstrap replicates (+BS brl). Identical sequences were not omitted for the analysis. Parsimony under dynamic homology was performed using POY v5.1.2 (Wheeler et al. 2015) for 9 gap:transversion:transition Sankoff matrices ranging from 1:1:1 to 16:4:1 (for a detailed procedure, see Schneider & D'Haese 2013). All trees were visualized using Figtree v1.4.2 (Rambaut & Drummond 2010).

Results

The genus *Acerentuloides* is characterized by 2 pairs of A-setae on the mesonotum and metanotum; 3 setae on the 2nd and 3rd pair of abdominal legs, of which the apical median seta is minute; well developed labial palpus with terminal tuft of setae and broadened sensillum; maxillary gland with small, smooth, globular vesicle or granulated appendix on the calyx and with several small globules in distal part; some setae modified as short, thickened sensilla (sd5 on head; β1 and δ4 on foretarsus; setae P4 on metanotum, M2 on prosternum, and A2 on thoracic sterna; and accessory setae on tergites and sternites I–VI); 6 setae on sternite VIII; anterior position of seta P3 on abdominal tergites II–VI; developed striate band with clearly visible parallel-sided striae. Foretarsal sensillum t1 is claviform, sensillum t3 is long and lancet-like (Bonet & Tuxen 1960) or finger-like, sensillum b' is present, the base of sensillum d is closer to base of c than to base of e, and the position of sensillum a' is close to the base of t1.

Acerentuloides bernardi Shrubovych **sp. nov.,** 2016 (Figs. 1–34; Table 2)

TYPE MATERIAL

The type materials were deposited in the collection of the Institute of Systematics and Evolution of Animals of the Polish Academy of Sciences (ISEA), Cracow, Poland, and in the collection of the State Museum of Natural History (SMNH) of the National Academy of Sciences of Ukraine, L'viv, Ukraine.

HOLOTYPE 1 female (ISEA 6647) USA: Indiana, Oakland City, Hugh Boyd, Patoka River National Wildlife Refuge and Management Area, 9-V-2008, latitude 38.3539°N, longitude 87.3156°E, elevation 133 m, deciduous growth with dominant black locust tree (*Robinia pseudoacacia* L.; Fabaceae) and red oak, sample of litter and rhizosphere, collected by J. Tajovský & J. Frouz.

PARATYPES 2 females (ISEA 6648, SMNH 89.1), data same as holotype.

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Table 1. List of investigated proturans and accession numbers of gene sequences used in the phylogenetic analysis (a dash indicates that the gene sequence was not available).

Species name[a]	*28S rDNA* D3–D6	*28S rDNA* D1–D2	*COI*	*18S rRNA*	Family	Subfamily
Proturentomon chinense	EU557257	EU557257	HQ882814	EU557244	Protentomidae	Protentominae
Neocondeellum brachytarsum	EU557259	EU557259	HQ882816	EU557245	Protentomidae	Condeellinae
Neocondeellum dolichotarsum	EU557258	EU557258	HQ882815	—	Protentomidae	Condeellinae
Hesperentomon nanshanensis	—	—	KF530828	—	Hesperentomidae	Hesperentominae
Hesperentomon pectigastrulum	EU557255	EU557255	HQ882812	EU557242	Hesperentomidae	Hesperentominae
Hesperentomon yangi	—	—	KF530824	—	Hesperentomidae	Hesperentominae
Acerella muscorum	HQ882811	HQ882811	HQ882825	HQ882809	Acerentomidae	Acerellinae
Acerella muscorum KJ101608	—	—	KJ101608	—	Acerentomidae	Acerellinae
Acerentomon carpaticum PROTA003	PROTA003-15	PROTA003-15	PROTA003-15	—	Acerentomidae	Acerentominae
Acerentomon carpaticum PROTA006	PROTA006-15	PROTA006-15	PROTA006-15	—	Acerentomidae	Acerentominae
Acerentomon carpaticum PROTA007	PROTA007-15	PROTA007-15	PROTA007-15	—	Acerentomidae	Acerentominae
Acerentomon dispar PROTA001	PROTA001-15	PROTA001-15	PROTA001-15	—	Acerentomidae	Acerentominae
Acerentomon dispar PROTA009	PROTA009-15	PROTA009-15	PROTA009-15	—	Acerentomidae	Acerentominae
Acerentomon dispar PROTA023	PROTA023-15	PROTA023-15	PROTA023-15	—	Acerentomidae	Acerentominae
Acerentomon dispar PROTA024	PROTA024-15	PROTA024-15	PROTA024-15	—	Acerentomidae	Acerentominae
Acerentomon dispar PROTA025	PROTA025-15	PROTA025-15	PROTA025-15	—	Acerentomidae	Acerentominae
Acerentomon italicum PROAT052	PROAT052-12	PROAT052-12	PROAT052-12	—	Acerentomidae	Acerentominae
Acerentomon maius PROAT001	PROAT001-12	PROAT001-12	PROAT001-12	—	Acerentomidae	Acerentominae
Acerentomon maius PROAT005	PROAT005-12	PROAT005-12	PROAT005-12	—	Acerentomidae	Acerentominae
Acerentomon maius PROAT017	PROAT017-12	PROAT017-12	PROAT017-12	—	Acerentomidae	Acerentominae
Acerentomon maius PROAT024	PROAT024-12	PROAT024-12	PROAT024-12	—	Acerentomidae	Acerentominae
Acerentomon microrhinus HQ8828	HQ882810	HQ882810	HQ882819	HQ882808	Acerentomidae	Acerentominae
Acerentomon microrhinus JQ728012	—	—	JQ728012	—	Acerentomidae	Acerentominae
Acerentomon christiani PROAT026	PROAT026-12	PROAT026-12	PROAT026-12	—	Acerentomidae	Acerentominae
Acerentomon christiani PROAT027	PROAT027-12	PROAT027-12	PROAT027-12	—	Acerentomidae	Acerentominae
Acerentomon christiani PROAT034	PROAT034-12	PROAT034-12	PROAT034-12	—	Acerentomidae	Acerentominae
Acerentomon *christiani* PROAT035	PROAT035-12	PROAT035-12	PROAT035-12	—	Acerentomidae	Acerentominae
Acerentomon christiani PROAT036	PROAT036-12	PROAT036-12	PROAT036-12	—	Acerentomidae	Acerentominae
Acerentomon christiani PROAT040	PROAT040-12	PROAT040-12	PROAT040-12	—	Acerentomidae	Acerentominae
Acerentomon christiani PROAT055	PROAT055-12	PROAT055-12	PROAT055-12	—	Acerentomidae	Acerentominae
Acerentomon christiani PROAT064	PROAT064-12	PROAT064-12	PROAT064-12	—	Acerentomidae	Acerentominae
Acerentomon *christiani* PROAT071	PROAT071-12	PROAT071-12	PROAT071-12	—	Acerentomidae	Acerentominae
Acerentomon spgr *microrhinus* PROAT022	PROAT022-12	PROAT022-12	PROAT022-12	—	Acerentomidae	Acerentominae
Acerentomon sp JX963616	—	—	JX963616	—	Acerentomidae	Acerentominae
Filientomon takanawanum	EU557261	EU557261	HQ882822	EU557249	Acerentomidae	Acerentominae
Huashanentulus huashanensis	EU557262	EU557262	HQ882821	EU557248	Acerentomidae	Acerentominae
Yamatentomon guoi YYH45	—	—	JQ846460	—	Acerentomidae	Acerentominae
Yamatentomon guoi YYH46	—	—	JQ846461	—	Acerentomidae	Acerentominae
Yamatentomon guoi YYH47	—	—	JQ846462	—	Acerentomidae	Acerentominae
Acerentulus charrieri cs59 ABPE002-17	ABPE002-17	—	JQ411216	ABPE002-17	Acerentomidae	Berberentulinae
Acerentulus charrieri cs64 ABPE003-17	ABPE003-17	—	JQ411217	—	Acerentomidae	Berberentulinae
Acerentulus confinis p029 ABPE004-17	—	ABPE004-17	ABPE004-17	ABPE004-17	Acerentomidae	Berberentulinae
Acerentulus noeli csp06 ABPE005-17	ABPE005-17	ABPE005-17	KJ210840	ABPE005-17	Acerentomidae	Berberentulinae
Acerentulus noeli csp13 ABPE006	—	—	KJ210841	—	Acerentomidae	Berberentulinae

[a]Extension after the species name corresponds to the specimen voucher code.

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Table 1. (Continued) List of investigated proturans and accession numbers of gene sequences used in the phylogenetic analysis (a dash indicates that the gene sequence was not available).

Species name[a]	*28S rDNA* D3–D6	*28S rDNA* D1–D2	*COI*	*18S rRNA*	Family	Subfamily
Acerentulus noeli csp14 ABPE007-17	ABPE007-17	—	KJ210842	— aExtension after the species name corresponds to the specimen voucher code.	Acerentomidae	Berberentulinae
Acerentulus pyreneicus csp10 ABPE008-17	ABPE008-17	—	KJ210838	ABPE008-17	Acerentomidae	Berberentulinae
Acerentulus pyreneicus csp11	—	—	KJ210839	—	Acerentomidae	Berberentulinae
Acerentulus sinensis	EU557260	EU557260	HQ882820	EU557247	Acerentomidae	Berberentulinae
Andinentulus rapoporti csp16 ABPE009-17	—	ABPE009	KJ395311	—	Acerentomidae	Berberentulinae
Andinentulus rapoporti csp19 ABPE010-17	ABPE010-17	ABPE010-17	KJ395312	ABPE010-17	Acerentomidae	Berberentulinae
Andinentulus rapoporti csp20 ABPE011-17	—	—	KJ395313	—	Acerentomidae	Berberentulinae
Baculentulus densus	EU376049	EU376049	—	—	Acerentomidae	Berberentulinae
Baculentulus tienmushanensis	EF192433	EF192433	HQ882817	AY037169	Acerentomidae	Berberentulinae
Gracilentulus maijiawensis	EF192435	EF192435	HQ882818	EU557246	Acerentomidae	Berberentulinae
Nosekiella sinensis	EU557263	EU557263	HQ882823	EU557251	Acerentomidae	Nipponentominae

[a]Extension after the species name corresponds to the specimen voucher code.

DESCRIPTION

Habitus typical for members of Acerentomidae (Fig. 24). Head setae short, cephalic seta l3 setiform (Figs. 1 and 2), seta sd5 thick, sensilliform (Figs. 1 and 3), setae sd4 and d6 absent, length ratio of posterior setae d7:sd7 as 1.0:1.5 (Fig. 1). Pseudoculus circular, with short posterior extension, PR = 14 (Fig. 2). Maxillary palpus apically with tuft of setae and 4 single setae, basal sensilla slender, subequal in length (Fig. 4). Labial palpus well developed with 4-branched apical tuft and sausage-shaped basal sensillum (Figs. 5 and 25). Maxillary gland with small elongated and weakly granulated calyx and small globular vesicles on calyx, long posterior filament with row of small globules posteriorly ending in bilobed dilation, CF = 3.7 (Fig. 6).

Foretarsus with sensillum b' present, t1 claviform, t3 long and finger-like, all other sensilla slender and parallel-sided (Figs. 9, 10, and 26). Sensillum a reaching base of t2. Sensillum b longer than c, extending past base of sensillum e. Sensillum c reaching base of sensillum e. Sensillum b inserted proximally to c, sensillum d inserted between sensilla c and t2. Sensillum a' short, extending past base of t2 (Fig. 26). Sensillum b' reaching base of sensillum c', apex of sensillum c' not reaching base of claw. Length formula of sensilla: t1 < t3 < (a' = b') < (g = c') < (c = t2) < e < (a = d) < b < f. Setae β1 and δ4 modified, sensilliform, shorter than other δ-setae (length of β1 = 4 µm, δ4 = 6 µm). Pores on foretarsus near base of sensillum t3 and between bases of sensillum c and seta α3. Claw short, without inner tooth, empodial appendage short. BS = 0.6, TR = 3.6, EU = 0.12.

Formula of chaetotaxy given in Table 2. Setae on nota strongly differing in length. Pronotal seta 1 twice the length of seta 2 (Fig. 11). Meso- and metanotal setae P1a and P2a very short, oblong (Fig. 27), P2a situated close to P3, seta P5 gemmate (Figs. 11 and 12). Length ratio of mesonotal setae P1:P2 = 1.0:1.6. Seta P4 on metanotum a short sensillum, length 3 µm (Fig. 12). Meso- and metanota with sl and al pores (Figs. 11 and 12). Thoracic sterna without pores. Setae A2 and M2 on prosternum and seta A2 on thoracic sterna short, sensilliform; length of prosternal A2 = 3 µm, of prosternal M2 = 1 µm (Figs. 28 and 29), of meso- and metasternal A2 = 1 µm (Figs. 13–15).

Setae A5 and P2a on tergite I, setae P2a and P4a on tergites II–VI short and sensilliform, their length 3 µm (Figs. 16, 17, and 30). Accessory setae P1a, P2a, and P4a on tergite VII setiform, longer, length 4 µm (Fig. 18). Seta P3 on tergites II–VI inserted anteriorly to other setae of P-row (Fig. 17), P3 on tergites I and VII level with other setae of P-row (Figs. 16 and 18). Tergites VI–VII with connecting line in anterior part (Figs. 17 and 18). Pores psm present on tergites I–VII between setae P1 and P2, and close to seta P1a on tergite VII, al pores on tergites II–VII, psl pores on tergite VI only (Figs. 16–18).

Abdominal legs with 4, 3, 3 setae. Subapical seta on 2nd and 3rd pairs of abdominal legs nearly twice the length of apical lateral seta (21 and 12 µm, respectively). Apical median seta minute, length 1 µm (Figs. 21 and 31). Accessory setae P1a on sternites I–VI sensilliform, on sternite VII setiform, short, P1a length on sternites I–VII 3 µm (Figs. 20, 22, 23, and 31–33). Sternites VI–VII with a connecting line in anterior region (Figs. 22 and 23). Sternites II–V with asymmetrical spsm pore (Fig. 20). Sternite VI with spsm pores, 2 near each other and 1 close to base of seta P1 (Figs. 22 and 32); sternite VII with spm pore (Fig. 23) or in 1 paratype with asymmetrical spsm pore between setae P1 and P1a (Fig. 33).

Abdominal segment VIII with well-developed striate band (Figs. 19 and 34). Pore psm without accompanying teeth (Fig. 19). Posterior margin of sternite VIII and laterotergites smooth (Figs. 23 and 34). Comb on tergite VIII with 8–9 short teeth (Fig. 7). Sternites IX–XII with smooth hind margin; setae 1 about one-third length of setae 2 (Figs.

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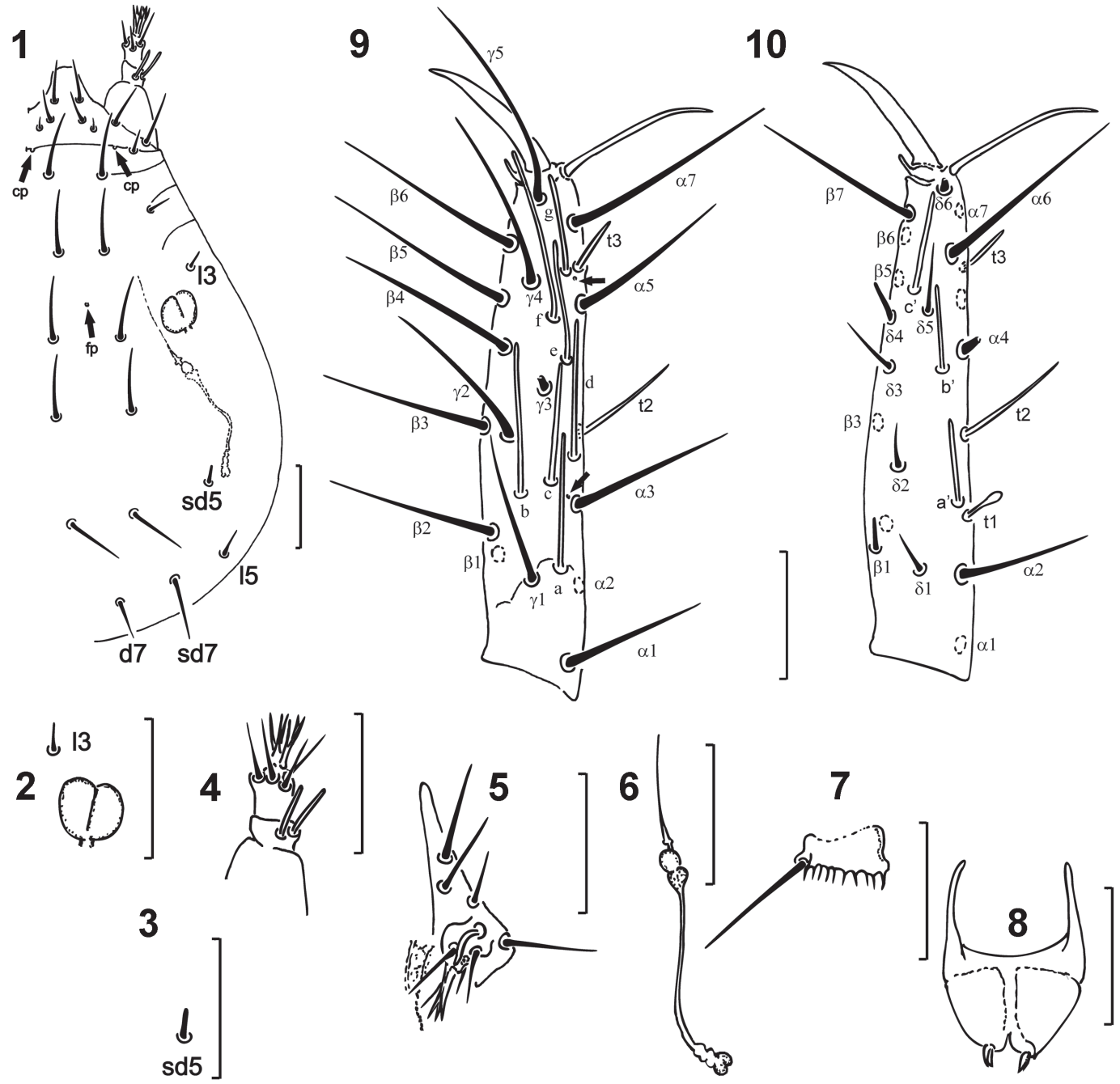

Figs. 1–10. *Acerentuloides bernardi* **sp. nov.** 1. Head, right side; 2. pseudoculus with seta l3; 3. cephalic seta sd5; 4. maxillary palpus; 5. labial palpus; 6. maxillary gland; 7. comb; 8. female squama genitalis; 9. foretarsus, exterior view; 10. foretarsus, interior view. Arrows indicate pores (cp = clypeal pore, fr = frontal pore). Scale bars: 20 µm.

23 and 34). Segment XII with medial pore on dorsal lobe and pair of sternal anterolateral pores.

Female squama genitalis with short, forked acrostyli (Fig. 8). Males unknown.

Body measurement (3 females): body length 1,100 µm; head 130–134 µm; pseudoculus 9 µm, lever 1 µm; posterior part of maxillary gland 35 µm; head setae d7 = 11 µm, sd7 = 16 µm, l3 = 6 µm; pronotal setae 1 = 25 µm, 2 = 12 µm, mesonotal setae P1 = 19–20 µm, P1a = 1 µm, P2 = 30–31 µm; foretarsus 90 µm, claw 25 µm, empodial appendage 3 µm.

ETYMOLOGY

The new species is cordially dedicated to our colleague and eminent zoologist, Prof. Dr. Ernest C. Bernard.

REMARKS

The habitus of the new species is very similar to *A. confinis* (Figs. 24 and 35). The species is characterized by absence of seta sd4 and additional seta d6 on head, absence of seta P1a on abdominal tergites I–VI, presence of seta Pc on sternite VII, short sensillum a reaching to

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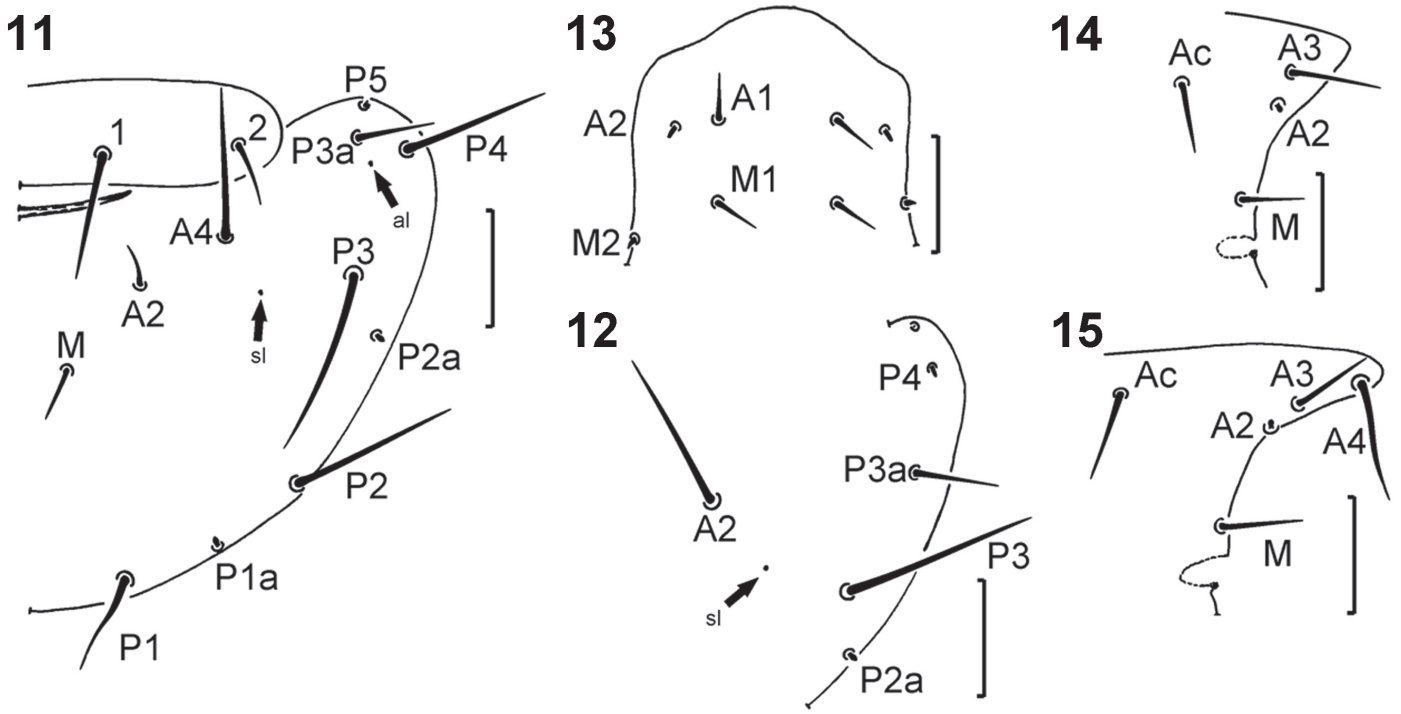

Figs. 11–15. *Acerentuloides bernardi* **sp. nov.** 11. Pronotum and mesonotum, right side; 12. lateral part of metanotum; 13. anterior part of prosternum; 14. anterolateral part of mesosternum; 15. anterolateral part of metasternum. Arrows indicate pores (al = tergal anterolateral, sl = tergal sublateral). Scale bars: 20 µm.

the base of sensillum t2, long foretarsal sensilla b and f, sensillum c shorter than b, short and slender sensilla a', b' and c', and presence of psl pores on tergite VI only. *Acerentuloides bernardi* **sp. nov.** is similar to *A. americanus* in the shape of the maxillary gland, presence of a minute apical medial seta on abdominal legs II and III, shape of the comb and the length of foretarsal setae a, b' and c' (Ewing 1921, 1940; Bonet & Tuxen 1960). The new species differs in possessing seta Pc on sternite VII and in the shape of foretarsal sensillum t3 (lancet-like in *A. americanus*, finger-like in the new species). Foretarsal sensillum b is longer than c and clearly surpasses the base of seta γ3 in the new species, whereas in *A. americanus* sensillum b is shorter and does not reach the base of γ3; in Canadian specimens of *A. americanus* sensilla b and c are of equal length (Nosek & McEwan Kevan 1984) and sensillum a' is longer and extends past the base of sensillum t2. In addition, the new species has a smooth, globular vesicle on the maxillary gland, whereas Canadian specimens of *A. americanus* have a granulated appendix on the calyx (Nosek & McEwan Kevan 1984).

MOLECULAR ANALYSIS

Unfortunately, DNA was not retrieved from specimens of *A. bernardi* **sp. nov.** However, the sequencing of *A. confinis*, a species from a closely related genus, was successful. A 658 bp fragment of the *COI* gene (DNA barcode) as well as 1,541 bp of the *18S rRNA* and 971 bp of the *28S rDNA* D1–D2 were amplified and sequenced from a specimen collected in the USA. To complete the *COI* data sets previously published (Shrubovych et al. 2012, 2014b, 2014c), other *Acerentulus* and *Andinentulus* species were amplified for *18S rRNA*: *Acerentulus charrieri* Shrubovych, Schneider & D'Haese, 2012, *A. noeli* Shrubovych, Schneider & D'Haese, 2014., *A. pyreneicus* Shrubovych, Schneider & D'Haese, 2014, and *Andinentulus rapoporti* (Condé, 1963); *28S rDNA* D1–D2: *A. noeli* and *Andinentulus rapoporti* (2 different specimens); *28S rDNA* D3–D6: *A. charrieri* (2 different specimens), *A. noeli* (2 different specimens), *A. pyreneicus*, and *Andinentulus rapoporti*. The sequences were deposited in BOLD under accession numbers ABPE002-17-ABPE011-17 (see Table 1).

The concatenated data (*COI*, *18S rRNA*, *28S rDNA* D1–D2, and *28S rDNA* D3–D6) consist of a total alignment length of 4,438 bp for 55 terminal taxa (27 species). The ML inference and direct optimization parsimony phylogenies all showed consistent agreement among topologies. The only real difference is the position of *Nosekiella+Huashanentulus* sister group of *Acerella* in the ML analysis and sister group of the rest of Acerentominae in parsimony. Here we show the ML tree (Fig. 41).

Discussion

Tuxen created the genus *Amerentulus* Tuxen, 1963 (currently *Acerentuloides* [Ewing, 1921]) on the basis of 2 differentiating peculiarities: the minute apical medial setae on 2nd and 3rd pairs of abdominal legs and the presence of a distal row of globules on the maxillary gland. The species described in the present paper is characterized by the absence of cephalic setae sd4, which is present in all *Acerentulus* species (see Fig. 36, American specimen of type species *A. confinis* [Berlese, 1908]). So far, this characteristic has been observed only in the monotypic genera *Podolinella* Szeptycki, 1995 and *Maderentulus* Tuxen, 1963 (Tuxen 1982, fig. 1). Szeptycki (1995) stated this characteristic in his generic diagnoses. Therefore, the absence of this seta could be an additional morphological character for *Acerentuloides* or perhaps only for *A. bernardi* **sp. nov.** The genus *Podolinella* differs from *Acerentuloides* in that abdominal legs II and III have only 2 setae and the labial palpus is reduced to a sensillum and 3 setae. *Maderentulus* differs from *Acerentuloides* in the shape of abdominal legs II and III with 2 setae and shape of foretarsal sensilla t1 (filiform) and t3 (cylindrical). The new species is characterized by having a finger-like foretarsal sensillum t3, which differs from the leaf-like shape of this sensillum in *Acerentulus* species, such as in American specimens of *A. confinis* (Fig. 37).

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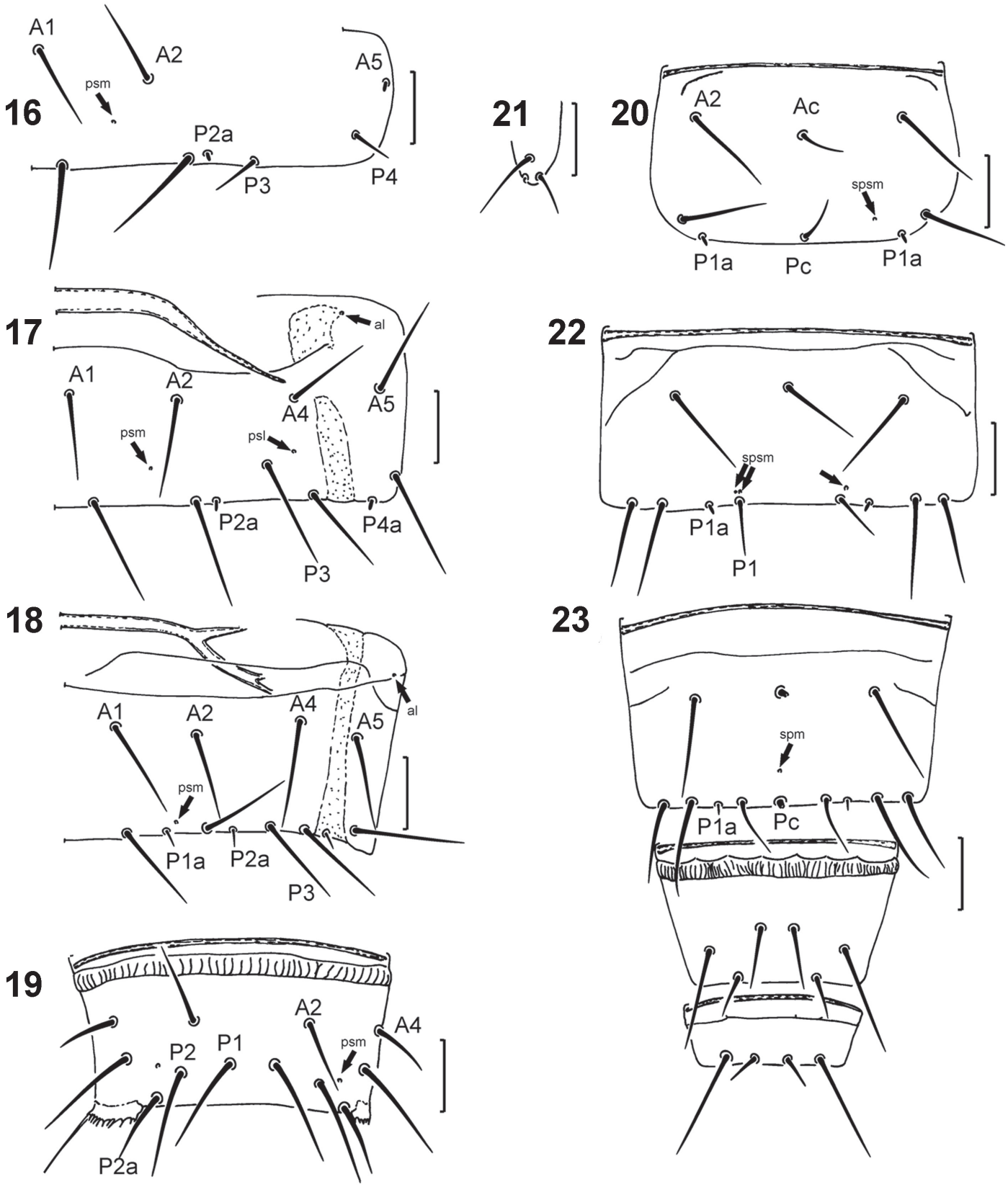

Figs. 16–23. *Acerentuloides bernardi* **sp. nov.** 16. Tergite I, right side; 17. tergite VI, right part; 18. tergite VII, right part; 19. tergite VIII; 20. sternite II; 21. abdominal leg of sternite II; 22. sternite VI; 23. sternites VII–IX. Arrows indicate pores (psm = tergal posterosubmedial, psl = tergal posterosublateral, spsm = sternal posterosubmedial, spm = sternal posteromedial). Scale bars: 20 µm.

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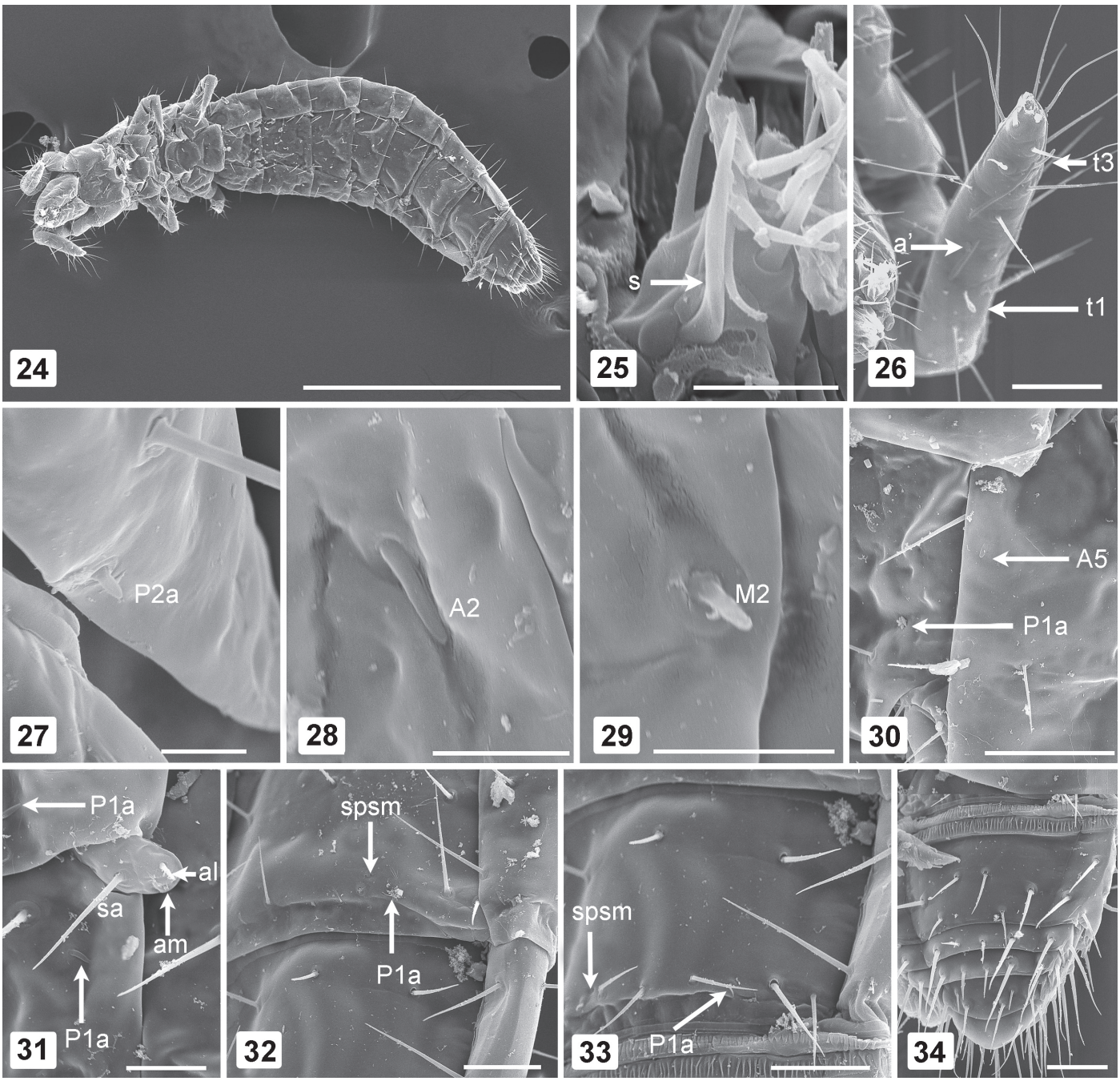

Figs. 24–34. *Acerentuloides bernardi* **sp. nov.** scanning electron microscopy photographs. 24. Habitus; 25. labial palp (apical tuft with 4 setae broken; s = labial sensillum); 26. foretarsus, interior view: sensilla t1, t3 and a'; 27. modified seta P2a on mesonotum; 28. modified seta A2 on prosternum; 29. modified seta M2 on prosternum; 30. modified seta A5 on tergite I and P1a on sternite I; 31. abdominal leg on segment III (sa = subapical, am = apical medial, al = apical lateral setae; apical lateral seta is broken); 32. hind margin of sternite VI; 33. hind margin of sternite VII; 34. sternites VIII–XII. Scale bars = 300 µm (Fig. 24), 4 µm (Figs. 25 and 27–29), 10 µm (Fig. 31), and 20 µm (Figs. 26, 30, and 32–34).

Porotaxy of the new species is typical for species of *Acerentulus*. The presence of psl pores on tergite VI and absence on tergite VIII may be specific features of *A. bernardi* **sp. nov.**, but the porotaxy of *Acerentulus* species is not well known. The porotaxy of *A. americanus* was not described, but Bonet & Tuxen (1960, pp. 283, 285) identified accessory setae P1a on tergites anterior to the posterior setae, exactly where psm pores are located in the new species. Furthermore, Tuxen (1964, p. 225) confirmed that these were dermal glands, not setae.

The genus *Acerentuloides* belongs to the group of Berberentulinae genera sensu Szeptycki (2007) that possess 3 setae on abdominal legs, such as *Acerentulus* Berlese, 1908, *Andinentulus* Tuxen, 1984, *Australentulus* Tuxen, 1967, *Brasilidia* Nosek, 1973, *Najtentulus* Szeptycki & Weiner, 1997, and *Fjellbergella* Nosek, 1978. Within this group *Acerentulus* and *Najtentulus* are characterized by a well developed labial palpus with an apical tuft of setae (Fig. 38), 6 setae and well-developed striate band on sternite VIII (Fig. 39), and modified sensilliform seta P4 on metanotum (Fig. 40) (Szeptycki & Weiner 1997; Shrubovych et al. 2012, 2014b). *Australentulus* also has a well-developed striate band, but differs in possessing reduced labial palpi and 4 setae on sternite VIII. *Andinentulus*, *Brasilidia*, and *Fjellbergella* also have 6 setae on

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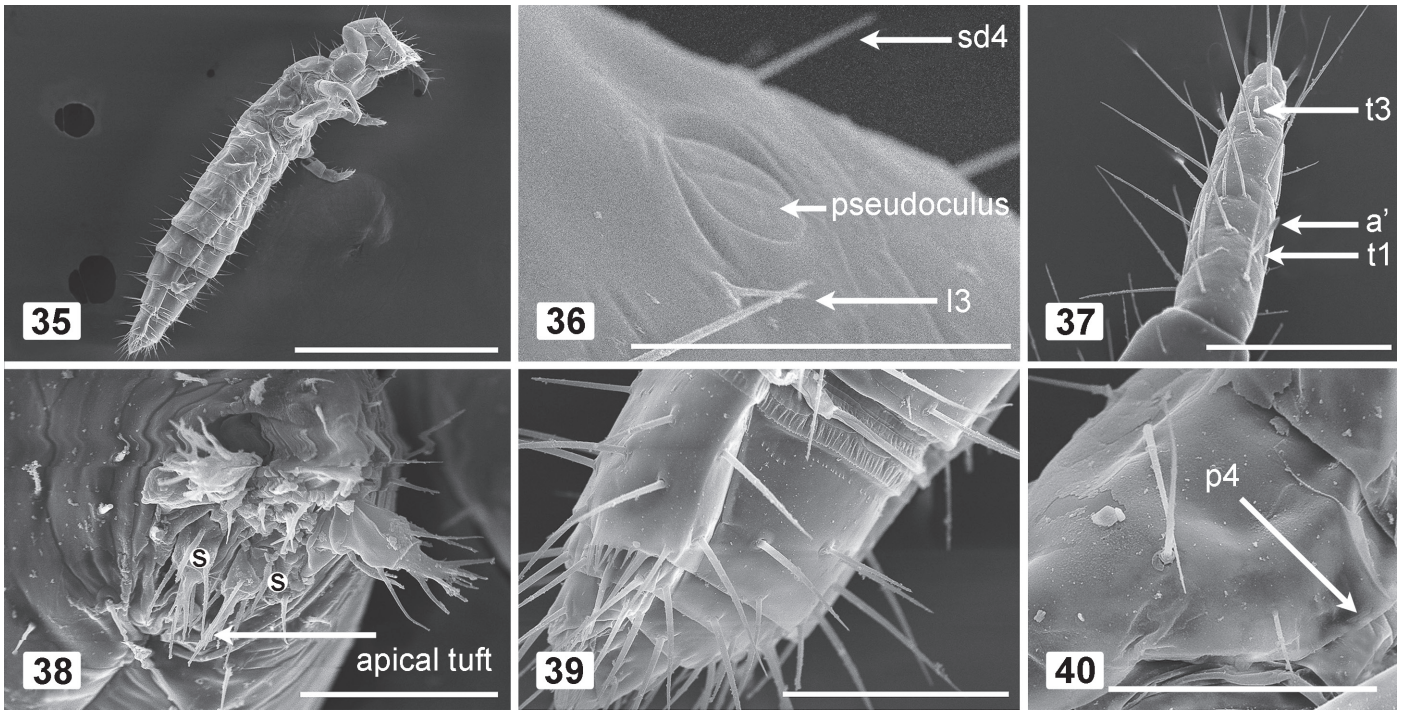
Table 2. Body chaetotaxy of *Acerentuloides bernardi* Shrubovych **sp. nov.**, 2016.

	Dorsal		Ventral	
Segment[a]	Formula	Setal composition	Formula	Setal composition
Th I	4	1, 2	4+4	A1, 2, M1, 2
			6	P1, 2, 3
Th II	6	A2, 4, M	5+2	Ac, 2, 3, M
	16	P1, 1a, 2, 2a, 3, 3a, 4, 5	4	P1, 3
Th III	6	A2, 4, M	7+2	Ac, 2, 3, 4, M
	16	P1, 1a, 2, 2a, 3, 3a, 4, 5	4	P1, 3
Abd I	6	A1, 2, 5	3	Ac, 2
	10	P1, 2, 2a, 3, 4	4	P1, 1a
Abd II–III	6	A1, 2, 5	3	Ac, 2
	14	P1, 2, 2a, 3, 4, 4a, 5	5	Pc, 1a, 2
Abd IV–V	6	A1, 2, 5	3	Ac, 2
	14	P1, 2, 2a, 3, 4, 4a, 5	8	P1, 1a, 2, 3
Abd VI	8	A1, 2, 4, 5	3	Ac, 2
	14	P1, 2, 2a, 3, 4, 4a, 5	8	P1, 1a, 2, 3
Abd VII	8	A1, 2, 4, 5	3	Ac, 2
	16	P1, 1a, 2, 2a, 3, 4, 4a, 5	9	Pc, 1, 1a, 2, 3
Abd VIII	6	A2, 4, 5	4	1, 2
	16	P1, 2, 2a, 3, 3a, 4, 4a, 5	2	P1a
Abd IX	12	1, 1a, 2, 2a, 3, 4	4	1, 2
Abd X	12	1, 1a, 2, 2a, 3, 4	4	1, 2
Abd XI	6	1, 3, 4	6	
Abd XII	9		6	

[a]Abd = abdominal segments, Th = thoracic segments.

sternite VIII, but differ from *Acerentuloides* by having a reduced labial palpus with 3 setae and a sensillum, and in reduction of the striate band to 2 lines without visible striae (Nosek 1973; Shrubovych & Bernard 2013; Shrubovych et al. 2014c).

Acerentuloides is morphologically close to the genera *Acerentulus* and *Najtentulus,* differing from them by the peculiar shape of the maxillary gland and strong reduction of the subapical median seta on abdominal legs II and III. Additionally, it differs from *Najtentulus* by the shape of the foretarsal sensillum t3 (finger-like in *Acerentuloides*, cylindrical in *Najtentulus*). The presence of a vesicle on the maxillary gland is found in only a few genera (*Vesiculentomon* Rusek, 1974, *Nosekientomon* (Nosek, 1977), *Callientomon* Yin, 1980, and *Acerella* Berlese, 1909), but they belong to other subfamilies of Acerentomidae (Tuxen 1964; Shrubovych et al. 2014a).



Figs. 35–40. *Acerentulus confinis* (Berlese, 1908), American specimen scanning electron microscopy photographs. 35. Habitus; 36. pseudoculus and cephalic setae sd4 and l3; 37. foretarsus, exterior view: sensilla t1, t3 and a'; 38. labial palpi with apical tuft of setae and basal sensillum (s); 39. sternite VIII; 40. modified seta P4 on metanotum. Scale bars = 300 µm (Fig. 35), 20 µm (Figs. 36 and 38), and 40 µm (Figs. 37, 39, and 40).

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Phylogenetic analyses, using both parsimony and ML, strongly support Acerentomidae as monophyletic (Fig. 41). Monophyly of Acerentomidae was already supported by the analysis of 67 morphological characters for 35 species of Acerentomata (Shrubovych 2014). The topology supports a monophyletic position of Berberentulinae. Berberentulinae is a subfamily including many genera (29 genera according to Szeptycki 2007) (Shrubovych 2014). In the present analyses, we included only 4 species of Berberentulinae, therefore more information about genetic data for morphologically differing genera of Berberentulinae is needed (such as *Amphientulus* Tuxen, 1981, *Australentulus* Tuxen, 1967, *Kenyentulus* Tuxen, 1961, *Silvestridia* Bonet, 1942, and *Tasmanentulus* Tuxen, 1984) to clearly confirm monophyly or even discuss family status for subfamily Berberentulinae.

Within Berberentulinae, species of *Acerentulus* form a separate and strongly supported taxon, which is sister group to a clade *Andinentulus* + *Baculentulus* + *Gracilentulus*. Within *Acerentulus*, *A. confinis* is the sister group of the remaining species of the genus. The Berberentulinae are sister group to a clade comprising all remaining subgroups proposed by Szeptycki (2007), i.e., Acerellinae + Nipponentominae + Acerentominae. Acerentominae are paraphyletic due to the position of the *Nosekiella* (Nipponentominae) as sister group to *Huashanentulus* (Acerentominae). François (2003) proposed to create a separate subfamily of Huashanentominae for genera *Huashanentulus* Yin, 1980 and *Sugaentulus* Imadaté, 1978 based on 2 apomorphies: absence of foretarsal sensillum b' and well-developed striate band. These characters also are native for other members of the subfamilies Nipponentomi-

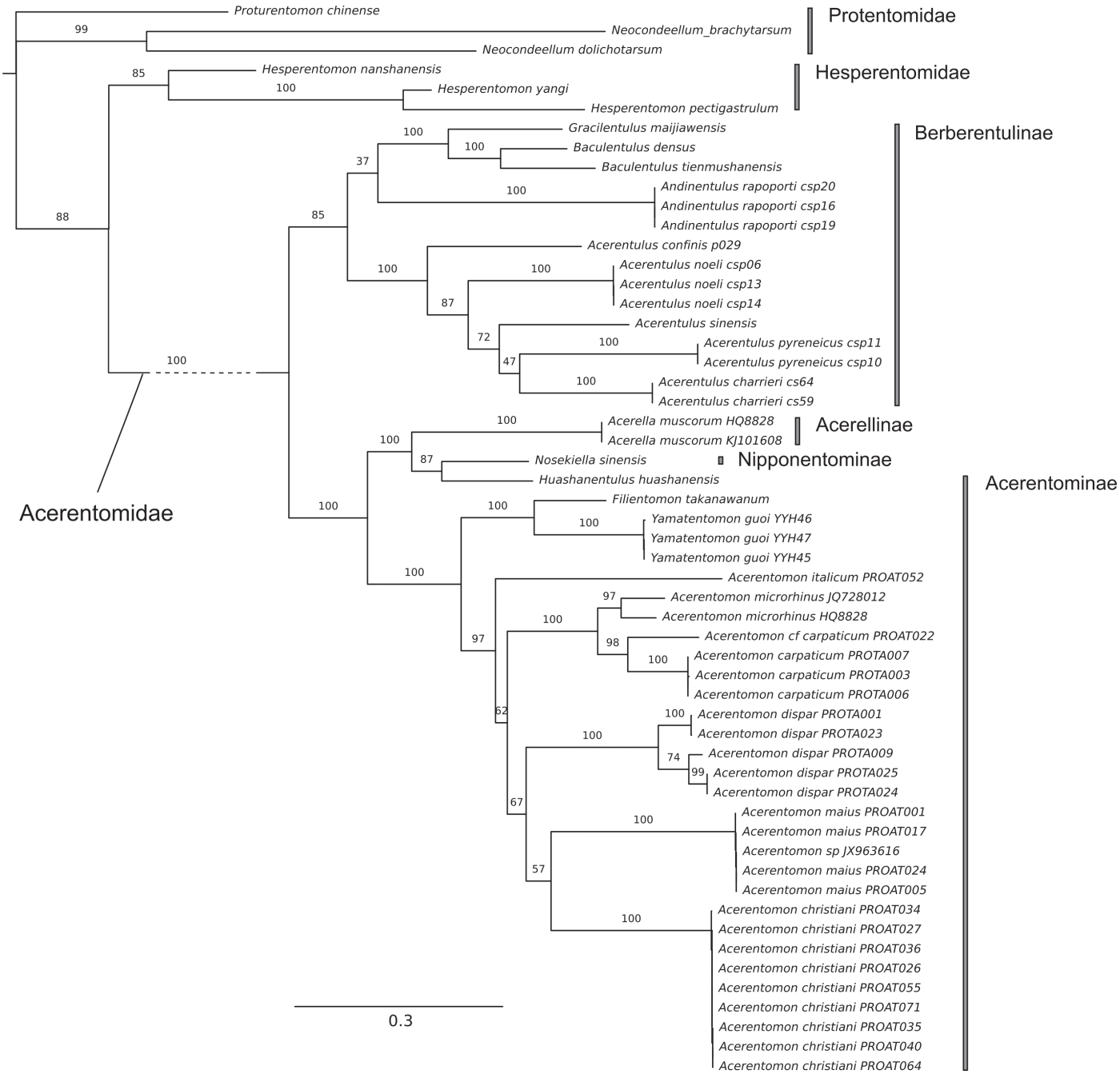

Fig. 41. Molecular phylogeny of Acerentomidae inferred from concatenated *COI*, *18S rRNA*, *28S rDNA* D1–D2, and *28S rDNA* D3–D6 sequences with maximum likelihood (ML). ML bootstrap values shown above the branches.

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nae, Acerellinae, and Acerentominae sensu Szeptycki (2007). Cladistic analysis, based on morphological characters, shows a separate position of *Huashanentulus* and *Sugaentulus* in a group of Acerellinae + Nipponentominae + Acerentominae genera (François 2003, p. 118, fig. 2, consensus tree). However, these genera do not form a joint clade on the consensus tree. Szeptycki (2007) included these genera in Acerentominae together with morphologically similar genera (Shrubovych & Rusek 2010). The genus *Huashanentulus* should be included in Nipponentominae according to our genetic results due to the strongly supported joint clade *Huashanentulus huashanensis* Yin, 1980 with *Nosekiella sinensis* Bu & Yin, 2008. We need additional genetic data for remaining nipponentomine and acerentomine genera to arrive at more comprehensive phylogenetic hypotheses.

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