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High diversity in the *Pseudechiniscus suillus–facettalis* complex (Heterotardigrada: Echiniscidae) with remarks on the morphology of the genus *Pseudechiniscus*

DARIA GROBYS[1*], MILENA ROSZKOWSKA[1,2], MAGDALENA GAWLAK[3], HANNA KMITA[1], ANDRZEJ KEPEL[4], MARTA KEPEL[4], IVAN PARNIKOZA[5,6], TOMASZ BARTYLAK[1] and ŁUKASZ KACZMAREK[2]

[1]*Department of Bioenergetics, Faculty of Biology, Adam Mickiewicz University, Poznań, Uniwersytetu Poznańskiego 6, 61–614 Poznań, Poland*
[2]*Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University, Poznań, Uniwersytetu Poznańskiego 6, 61–614 Poznań, Poland*
[3]*The Institute of Plant Protection-National Research Institute, Węgorka 20, 60–318 Poznań, Poland*
[4]*Polish Society for Nature Conservation 'Salamandra', Stolarska 7/3, 60–788 Poznań, Poland*
[5]*National Antarctic Scientific Center of Ukraine, Boulevard Tarasa Shevchenka 16, 01601 Kyiv, Ukraine*
[6]*Institute of Molecular Biology and Genetics of National Academy of Science of Ukraine, Kyiv, Ukraine*

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Pseudechiniscus is a morphologically homogeneous genus of tardigrades. The morphological features commonly used for species discrimination in this genus are the dorsal sculpture, the shape and number of dorsal plates and trunk appendages. Species of the *Pseudechiniscus suillus–facettalis* complex are one of the most challenging tardigrades to identify. All species are similar in their general appearance and all lack trunk appendages. Moreover, not only the nominal *Pseudechiniscus suillus*, but also other members of the *suillus–facettalis* complex have been insufficiently described. In our study, we examined several populations from the Northern and the Southern Hemispheres that could be traditionally attributed to *Pse. suillus*. These populations were analysed using integrative taxonomy – a combination of classical morphology and morphometry with molecular data. Besides the differences in the dorsal sculpture and morphometry, we also found species-specific differences in ventral sculpture, which were originally used for discrimination of *Pseudechiniscus* species. Moreover, we provide an extensive discussion on all morphological and morphometric differences used in *Pseudechiniscus* taxonomy and indicate main taxonomic problems with this genus. Finally, we redescribe the nominal *Pse. suillus* from Italy.

ADDITIONAL KEYWORDS: *COI* – ITS2 – *Pseudechiniscus suillus* – species complex – taxonomy – Tardigrada – ventral sculpture.

INTRODUCTION

The genus *Pseudechiniscus* was established by Thulin (1911) and later emended by Kristensen (1987) and Vecchi *et al.* (2016). It is characterized mainly by the presence of a pseudosegmental plate (psp) situated just before the terminal/caudal plate (cap), and by the presence of external, and internal buccal cirri and filamentous cirri *A*. Lateral and dorsal filaments or spines are most often absent or reduced in number and/or size. The dentate collar is generally absent on legs IV. *Pseudechiniscus* is a homogeneous taxon and most species are similar to each other. The morphological characters that are usually used in species differentiation are dorsal sculpture, shape and number of dorsal plates, and structure of dorsal appendages present mainly on the psp (e.g. see: Ramazzotti & Maucci, 1983). Recently, geometric morphometrics has been proposed as a useful complementary tool for use in the taxonomy of the genus *Pseudechiniscus* (Fontoura & Morais, 2011). At present, 40 species and subspecies are attributed to this genus. Five *Pseudechiniscus*

*Corresponding author. E-mail: grobysdaria@gmail.com

species were excluded from this genus and transferred to the new genus *Acanthechiniscus*, mainly because of the presence of lateral appendages and a dentate collar on legs IV (Vecchi *et al.*, 2016).

The species of the *Pseudechiniscus suillus–facettalis* complex are some of the most challenging tardigrades to identify. All species are highly similar morphologically and mainly characterized by the absence of dorsal and lateral appendages (except for cephalic appendages and cirri *A*). Moreover, not only the nominal species *Pse. suillus* (Ehrenberg, 1853), but also other members of the complex have been insufficiently described (see also: Tumanov, 2020). As a result, inadequate characters have been used to describe new taxa, and newly found specimens have been erroneously identified as *Pse. suillus* (for more details see the Discussion below).

In this study, several populations from Madagascar, Antarctica and Europe, which would traditionally be attributed to *Pse. suillus*, were examined. These populations were analysed using integrative taxonomy, which includes classical morphology and morphometry analysed by phase-contrast light microscopy (PCM) and scanning electron microscopy (SEM), as well as molecular data (*COI* and ITS2 nucleotide sequence analysis). Based on the ventral sculpture and DNA markers, we redescribe the nominal *Pse. suillus* from its type locality in the Italian Alps and attribute the remaining populations to five new species (which will be described in a subsequent work). We discuss the morphological characters important in *Pseudechiniscus* taxonomy, with a special emphasis on the ventral sculpture and the taxonomic position of some members of the genus *Pseudechiniscus*.

MATERIAL AND METHODS

Sample processing

Moss and lichen samples were collected in Antarctica (Galindez Island, Argentine Islands, maritime Antarctic), Italy (Aosta Valley and Trentino Province), Madagascar (Fianarantsoa Province) and Norway (Rogaland and Buskerud Provinces) between 2016 and 2019. All samples were packed in paper envelopes, dried at room temperature and delivered to the laboratory in the Department of Animal Taxonomy and Ecology at the Faculty of Biology, Adam Mickiewicz University, Poznań, Poland. Tardigrades were extracted from the samples and studied following the standard methods described in Dastych (1980) with modifications in Stec *et al.* (2015).

Microscopy and imaging

Specimens for light microscopy were mounted on microscope slides in a small drop of Hoyer's medium, prepared according to Ramazzotti & Maucci (1983) as in the English translation by Beasley (1995) and secured with a cover slip. The slides were then placed in an incubator and dried for two days at *ca*. 60 °C. Dried slides were sealed with a transparent nail polish and examined under an Olympus BX41 phase-contrast light microscope (PCM) equipped with an ARTCAM-300Mi digital camera (Olympus Corporation, Shinjuku-ku, Japan). In order to obtain clean and stretched specimens for scanning electron microscopy (SEM), tardigrades were rinsed several times with double distilled H_2O, before being put through a water/ethanol series (from 0% to 100% ethanol, with 10% increments). The specimens were then moved to an ethanol/acetone mixture (100% ethanol and 100% acetone in 1:1 proportion) and, finally, rinsed three times with 100% acetone. Transfer between solutions was achieved via small cages made out of short, plastic tubes closed at each end by a fine plastic mesh (40-µm grade). The dehydrated specimens were CO_2 -critical-point dried, transferred with an eyebrow hair mounted on a wooden stick to a SEM stub covered with a double-sided conductive tape, and sputter coated with a thin layer of gold. The prepared specimens were examined under high vacuum in a Hitachi S3000N Scanning Electron Microscope.

All figures were assembled in Corel Photo-Paint 2017 and Inkscape 0.92. For deep structures that could not be fully focused in a single PCM photograph, a series of two to ten images were taken every *ca*. 0.5 µm and then assembled into a single deep-focus image manually in Corel Photo-Paint 2017.

Morphometrics and morphological nomenclature

All measurements are given in micrometres (µm). Structures were measured only if their orientation was suitable. Body length was measured from the anterior extremity to the end of the body, excluding the hind legs. The terminology used in the descriptions, partially follows Kristensen (1987) and Tumanov (2020), but some new structure names are introduced in this study. Claw heights were measured from the base of the claw to the top of the branch. The *sp* index is the ratio of the length of a given structure to the length of the scapular plate (scp) expressed as a percentage (length of the structure × 100/scp length) (Dastych, 1999 and later proposed as the *psc* index by Fontoura & Morais, 2011). Configuration and arrangement of body appendages (chaetotaxy) is given according to

Gąsiorek *et al.* (2017). Genus abbreviations follow Perry *et al.* (2019).

Morphometric data were handled using the 'Echiniscoidea' v.1.2 template available from the Tardigrada Register (Michalczyk & Kaczmarek, 2013). Tardigrade taxonomy follows Guil *et al.* (2019).

Comparative material

Pseudechiniscus suillus–facettalis taxa from Costa Rica, Ecuador, Italy, Madagascar, Mongolia, Norway and Spitsbergen, identified using the key in Ramazzotti & Maucci (1983), were used as comparative material. Additionally, original descriptions and redescriptions of *Pse. beasleyi* Li, Wang & Yu, 2007, *Pse. clavatus* Mihelčič, 1955, *Pse. facettalis* Petersen, 1951, *Pse. jiroveci* Bartoš, 1963, *Pse. juanitae* de Barros, 1939, *Pse. megacephalus* Mihelčič, 1951, *Pse. mutabilis* (Murray, 1905), *Pse. suillus* (Ehrenberg, 1853), *Pse. suillus franciscae* de Barros, 1942 and *Pse. xiai* Wang, Xue & Li, 2018 were also considered (Ehrenberg, 1853; Murray, 1905; de Barros, 1939, 1942; Mihelčič, 1951, 1955; Petersen, 1951; Bartoš, 1963; Pilato & Lisi, 2006; Li *et al.*, 2007; Wang *et al.*, 2018).

Genotyping

Before DNA extraction, all specimens were preliminarily identified using PCM. Later, each specimen was placed individually in a 1.5-mL Eppendorf microcentrifuge tube in 20 µL of sterile MilliQ H_2O (MQ H_2O) and kept frozen at –80 °C until DNA isolation. DNA extraction was performed following the Chelex 100 resin (Bio-Rad) extraction method (Casquet *et al.*, 2012; Stec *et al.*, 2015), and tardigrade exoskeletons were recovered and mounted on permanent microscope slides to provide voucher specimens (hologenophores *sensu* Pleijel *et al.*, 2008).

Each specimen was incubated in 40 µL of 10% Chelex 100 resin solution in sterile MQ H_2O with the addition of 0.02 mg of Proteinase K (Genoplast) at 55 °C for 5 h with shaking (500 RPM, Eppendorf Thermomixer 5436) and the samples were occasionally centrifuged. Later the tubes were incubated at 70 °C for 15 min in order to inactivate Proteinase K. In the next step, 20 µL of sterile MQ H_2O was added to the samples and the tubes were centrifuged for 2 min at 8000 *g*. Then, *ca.* 40 µL of DNA extract (to the level of remaining Chelex beads at the bottom) was carefully transferred from each tube to a new 1.5-mL Eppendorf microcentrifuge tube. The tardigrade exoskeleton, present in a pellet after centrifugation, was extracted under stereomicroscope and then mounted in Hoyer's medium for further morphological analysis. The polymerase chain reaction (PCR) amplification was carried out for two DNA fragments differing in mutation rates: mitochondrial cytochrome *c* oxidase subunit I (*COI*) and nuclear internal transcribed spacer 2 (ITS2) in a total volume of 25 µL (see Table 1 for primers, Table 2 for the PCR cocktail recipes and Table 3 for the PCR programmes). The PCR products were examined by agarose gel electrophoresis (1% agarose) in the presence of ethidium bromide and verified by sequencing. Prior to sequencing, the PCR products were purified to improve their quality. In the case of *COI,* PCR products were purified by thermosensitive Exonuclease I and FastAP Alkaline Phosphatase (Fermentas, Thermo Scientific), and in the case of ITS2 (after excision from the gel) by NucleoSpin Gel and PCR Clean-up Kit (MARCHERY-NAGEL). The obtained PCR products were sequenced bidirectionally with BigDye Terminator v.3.1 by ABI Prism 3130XL Analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. The sequences were edited and manually checked against non-conservative alignments using BioEdit, v.7.0.5. (Hall, 1999). They were also analysed by Standard Nucleotide BLAST to check if they fulfilled the uniqueness criterion and submitted to GenBank (see the Results).

Comparative molecular analysis

Comparative molecular analysis was performed for the obtained *COI* sequences of the genus *Pseudechiniscus* deposited in GenBank [*Pse.* aff. *facettalis* (deposited in GenBank as *Pse. facettalis*): HM193415 from Jørgensen *et al.* (2011), JX683830–1 from Vicente *et al.* (2013a), FJ435811–2 from Guil & Giribet (2012) and *Pse*. aff.

Table 1. Primers used for amplification and sequencing of DNA fragments

DNA fragment	Direction	Code	Sequence (5'–3')	Reference
COI	Forward	bcdF01	CATTTTCHACTAAYCATAARGATATTGG	Dabert *et al.* (2010)
	Reverse	bcdR04	TATAAACYTCDGGATGNCCAAAAAA	Dabert *et al.* (2008)
	Forward	LCO 1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.* (1994)
	Reverse	HCO 2198	TAAACTTCAGGGTGACCAAAAAATCA	
ITS2	Forward	ITS2_Eutar_Ff	CGTAACGTGAATTGCAGGAC	Stec *et al.* (2018)
	Reverse	ITS2_Eutar_Rr	TGATATGCTTAAGTTCAGCGG	

Table 2. PCR cocktails used for the amplification of DNA fragments

Component	Additional note	Concentration
H_2O	MQ	–
buffer	5X Phusion HF Buffer; Thermo Scientific	1x
dNTPs	dNTP Mix; Thermo Scientific	200 µM
forward primer	*COI*: LCO 1490 for *Pse. suillus s.s.* and bcdF01 for other species ITS–2: ITS2_Eutar_Ff for all species	0.5 µM
reverse primer	*COI*: HCO 2198 for *Pse. suillus s.s.* and bcdR04 for other species ITS–2: ITS2_Eutar_Rr for all species	0.5 µM
polymerase	Phusion High–Fidelity DNA Polymerase; Thermo Scientific	0.02 U/µL
DNA	1–2 µL	–

Table 3. PCR programmes used for the amplification of *COI* and ITS2

	COI			ITS2		
Step	Cycles	Time (min:s)	Temp. (°C)	Cycles	Time (min:s)	Temp. (°C)
initial denaturation	–	05:00	98	–	05:00	98
denaturation	5	00:30	98	–	–	–
annealing		00:30	45		–	–
extension		01:00	72		–	–
denaturation	30	00:30	98	35	00:30	98
annealing		00:30	50		00:30	50
extension		01:00	72		01:00	72
final extension	–	07:00	72	–	07:00	72

facettalis: MK804898, *Pse.* aff. *suillus*: MK804899–908, *Pse*. aff. *xiai*: MK804894–7 from Cesari *et al.* (2020)] and several sequences deposited as *Echiniscus* spp. (KJ857005–8 from Velasco Castrillón *et. al*., 2015), which are in fact misidentified *Pseudechiniscus* sequences (see Results and Disscusion). Comparative molecular analysis for ITS2 was possible only for the sequences obtained in the present study, because no ITS2 sequences for *Pseudechiniscus* were available in GenBank at the time of this study. In the analysis, we only used sequences obtained from specimens from which we had complete information, i.e. the exoskeleton as well as the *COI* and ITS2 sequences. All sequences were aligned using the ClustalW Multiple Alignment tool (Thompson *et al.*, 1994), implemented in BioEdit. The analysis was performed for 30 (*COI*) and six (ITS2) nucleotide sequences. The genetic differential diagnosis between *Pse. suillus s.s.* and sequences from GenBank, as well as *Pseudechiniscus* sp. 1–5, were determined using p-distance for pairwise distance calculations. All positions with less than 95% site coverage were eliminated. The final dataset included 565 (*COI*) and 234 (ITS2) positions. Distance estimations were performed using MEGA 7 (Kumar *et al.*, 2016).

Molecular phylogenetic analysis by maximum likelihood method was performed in MEGA 7 using *COI* and ITS2 sequences. According to the Bayesian information criterion (BIC) the GTR+G+I substitution model (Nei & Kumar, 2000) was found to be the best-fit substitution model for the *COI* dataset, whereas T92+G (Tamura, 1992) was found to be the best-fit substitution model for the ITS2 dataset. The best-fit substitution model was calculated using an algorithm implemented in MEGA 7. The included codon positions were $1^{st}+2^{nd}+3^{rd}$+noncoding. The analysis involved 90 and 20 nucleotide sequences, respectively (see Supporting Information, S1). The phylogenetic analysis included also data from outgroups: some *Echiniscus* Schultze, 1840 and *Milnesium* Doyère, 1840 species, as well as *Acanthechiniscus islandicus* (Richters, 1904). The tree was rooted with *Milnesium*. There was a total of 511 (*COI*) and 404 (ITS2) positions in the final dataset. Trees obtained in MEGA 7 were manually modified in Corel Photo-Paint 2017 and Inkscape 0.92.

The phylogenetic analysis of ten combined *COI* and ITS2 sequences (together 1014 positions, 616 of *COI* + 398 of ITS2) using GTR+G and HKY+G models (Hasegawa *et al.*, 1985), respectively, was conducted with MrBayes v.3.2.6 (Ronquist & Huelsenbeck,

2003). Some *Echiniscus* [*Ech. testudo* (Doyère, 1840) and *Ech. tristis* Gąsiorek & Kristensen, 2018] and *Mil. berladnicorum* Ciobanu, Zawierucha, Moglan & Kaczmarek, 2014 species were used as outgroups [*Ech. testudo:* MG025605+MG016456 from Gąsiorek *et al.* (2017); *Ech. tristis*: MN239903+MN275479 and MN239904+MN275479 from Bartylak *et al.* (2019); *Mil. berladnicorum*: KT951659+KT951662 from Morek *et al.* (2016)]. The tree was rooted on *Mil. berladnicorum*. Four Monte Carlo Markov chains were run for 10 000 000 generations, with sampling every 100 generations and diagnosis every 1000 generations (the first 25% trees were discarded as 'burn-in'). That gave us a 50% majority rule consensus tree. The tree obtained by MrBayes v.3.2.6 was prepared in FigTree v.1.4.3 and manually modified in Inkscape 0.92.

RESULTS

MORPHOLOGY OF THE GENUS *PSEUDECHINISCUS*

Dorsal and ventral plates and sculpture

The dorsal plates in *Pseudechiniscus* are noticeably softer and more elastic in comparison to other genera of Echiniscidae. For example, *Echiniscus* has plates that are thicker and much less flexible. This feature is well visible in SEM, where the dorsal plates of *Pseudechiniscus* are often not well marked, concave, convex or deformed (Fig. 1A, B), whereas in *Echiniscus* the plates are always well marked and usually not deformed (Fig. 1C, D).

The arrangement of the dorsal plates in *Pseudechiniscus* is in accordance with the characteristics proposed by Kristensen (1987) and Tumanov (2020). However, we here describe some aspects of the plate arrangement in more detail. Typically, almost the entire dorsal side of the body is divided by a median longitudinal fold, which is less visible on median plates (m1, m2, m3) and absent on the caudal plate (cap) (Fig. 1A, asterisks). On the cephalic plate (cp), the characteristic W-shaped pattern is visible, dividing the plate into five smaller parts (giving the impression of faceting of the cp) (Fig. 2A, empty arrow). The scapular plate (scp) is divided by a transversal fold into two parts: an anterior part (Fig. 1A, ap) that is generally wider and a posterior part (Fig. 1A, pp) that is narrower. The wider part is always divided by a median longitudinal fold into two parts. The posterior part can also be divided by two lateral longitudinal folds and the mentioned median longitudinal fold, into four plate parts/subplates (Fig. 2A, asterisks). In *Pse. beasleyi*, such small plate parts/subplates are also visible in the anterior part of the scp (Li *et al.*, 2007). Additionally, on the lateral sides of the scp, plate-like structures are visible in PCM (but not visible under SEM), which are parts of scp separated from it by the refractions of scp (similar structures were, for example, observed also in *Barbaria ganczareki* (Michalczyk & Kaczmarek, 2007) and are probably also present in other Echinicidae). Plates m1, m2 and m3 can be divided or not (see: Tumanov, 2020). The paired plates s1, s2 can have some additional transversal and longitudinal folds, which can form visible ridges. Generally, the entire cap is concave

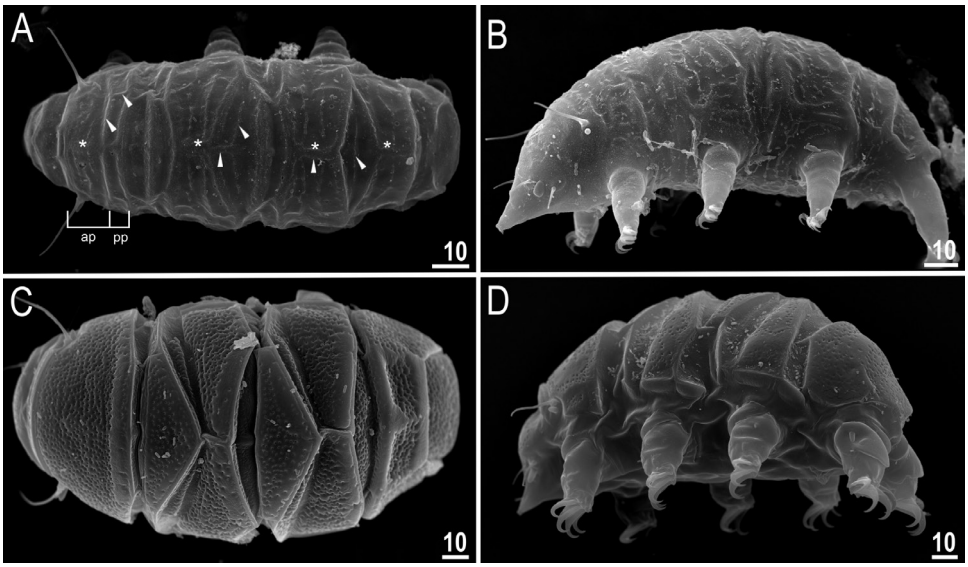

Figure 1. Comparison of dorsal plates in genera A, B – *Pseudechiniscus* and C, D – *Echiniscus.* Filled arrowheads indicate folds forming separate plate parts/subplates; asterisks indicate the median longitudinal fold; ap – anterior part of the scapular plate (scp); pp – posterior part of scp. All SEM. Scale bars in micrometres (μm).

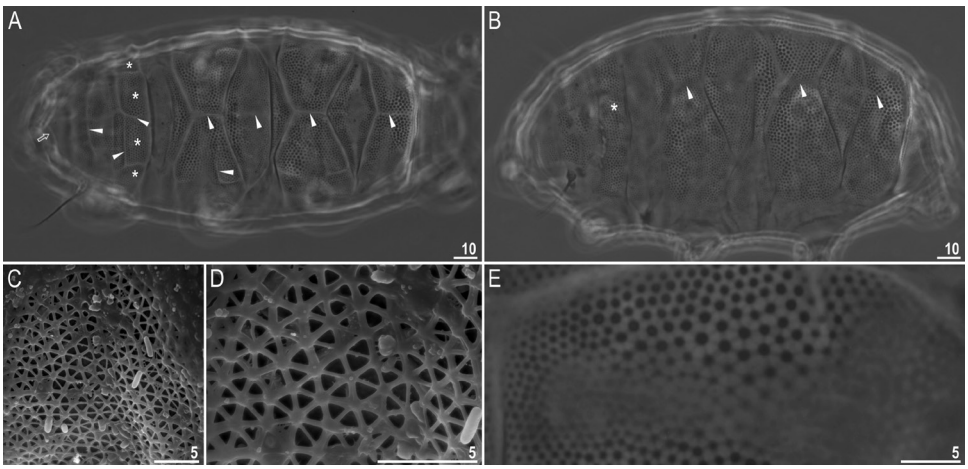

Figure 2. A, folds (arrowheads) and plate parts/subplates (asterisks) visible on the dorsal side in *Pseudechiniscus* sp. 2; thick slide (PCM). B, folds (arrowheads) and plate parts/subplates (asterisks) visible on the dorsal side in *Pseudechiniscus* sp. 2; thin slide (PCM). C–E, upper ends of cuticular pillars connected by striae in *Pseudechiniscus* sp. 2 (SEM and PCM, respectively). Scale bars in micrometres (μm).

and possesses two Y-shaped bifurcated ridges. Between these ridges, the cuticle is even more concave. The ridges, together with the clearly concave cuticle, give the impression of cap faceting, similar to that on cp. All of these folds forming separate plate parts/subplates are visible using PCM as white lines and with the SEM as ridges (Figs 1A, 2A, B, filled arrowheads), but the visibility of these folds (and, as a result, also the plate parts/subplates) is strongly related to the microscope slide preparation. In slides with a thick layer of mounting medium, where the specimens were not well flattened, the folds are clearly visible, whereas, in well-flattened specimens (thin microscope slides), these folds and plate parts/subplates are poorly visible or not at all visible (Fig. 2A, B, filled arrowheads). This can lead to serious problems with the correct interpretation of this morphological character. For example, in certain species examined in this paper, the number of visible plate parts/subplates located on the posterior margin of the scp can vary between zero to one in well-flattened specimens and up to four in specimens on microscope slides with a thick layer of mounting medium (Fig. 2A, B, asterisks).

Granulation on the ventral side of the body and on dorsal plates may be more or less spaced, and the granules can be smaller or larger. These granules are pillars of the endocuticle and support a thin epicuticle, which is an easily deformable layer. On the dorsal side, upper ends of cuticular pillars are connected by thin striae (which are probably present in all *Pseudechiniscus* taxa, but sometimes are delicate and not visible in PCM), which form a thin hexagonal pattern (Fig. 2C–E). On the ventral side, the striae are absent or not visible in PCM, but this needs to be confirmed in further studies. The granulation on the dorsal side is regular and the only differences are the sizes of the individual granules, presence or absence of striae (visible or not visible in PCM) and the spacing between granules. In contrast, on the ventral side this granulation forms a species-specific pattern, which is complex and difficult to describe in detail (see Fig. 3). This ventral pattern is always visible as convex in SEM in comparison to the remaining ventral cuticle. Apart from this pattern, more clearly marked fields, with coarser granulation (patches of granulation; PG) in comparison to the other types of ventral granulation, are also present on the ventral side. In contrast to the ventral pattern, they are concave when studied in SEM (Fig. 3).

These patches of granulation with different shapes may be present below the head, in line with legs I–III, between legs I and II, II and III, and III and IV, as well as above, below or around the gonopore (Fig. 3). Granulation on the patches located in line with the legs and close to the gonopore is always a little larger and, consequently, more visible than that on the other PGs. To distinguish these patches of granulation, we suggest naming them accordingly:

PG I – patch of granulation situated on the ventral side of the head.
PG II – situated in line with legs I.
PG III – situated between legs I and II.
PG IV – situated in line with legs II.
PG V – situated between legs II and III.
PG VI – situated in line with legs III.
PG VII – situated between legs III and IV.
PG $VIII_a$ $VIII_b$ or $VIII_g$ – situated above, below or around the gonopore, respectively.

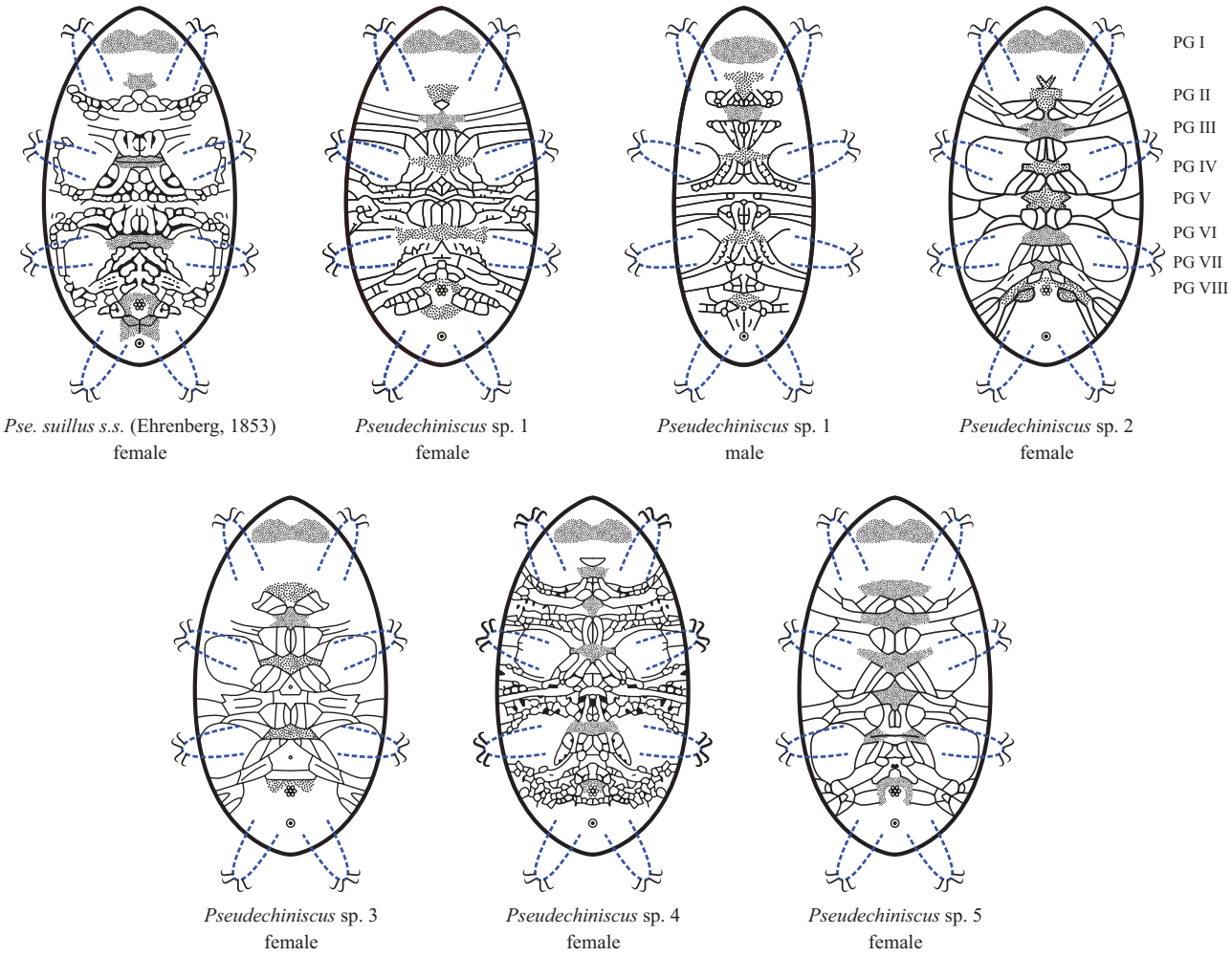

Figure 3. A schematic illustration of ventral patterns of the *Pseudechiniscus* species presented in this study. PG I–VIII, patches of granulation I–VIII. Scale bars in micrometres (μm).

For example, a formula of the arrangement of the PGs could be PG:I-II-III-V-VII-VIII$_g$, meaning that the PGs are present in all positions, except IV and VI, and that the PG VIII is situated around the gonopore.

Dorsal and lateral lobes, spines or filaments

Appendages are present mainly on the psp, but occasionally can also be present on other dorsal plates (*Pse. quadrilobatus* Iharos, 1969) and/or lateral side of the body [e.g. see: *Pse. conifer* (Richters, 1904), *Pse. n. novaezeelandiae* or *Pse. occultus* Dastych, 1980]. Lobes and spines are generally short or reduced, can be even or odd, and can have highly variable shapes, even in the same species, as seen, for example, in *Pse. nataliae* Biserov & Maucci, 1986, *Pse. n. novaezeelandiae*, *Pse. r. ramazzottii* Maucci, 1952 and *Pse. santomensis* Fontoura, Pilato & Lisi, 2010 (see also: Ramazzotti & Maucci, 1983; Biserov, 1986; Fontoura *et al.*, 2010). Longer spines or filaments on the lateral side of the body are present only in a few species; for example, in *Pse. pulcher* (Murray, 1910) or *Pse. transsylvanicus* Iharos, 1936.

Other morphological characters

The cirri *interni* and *externi*, as well as finger-like cephalic papillae, are present on the head. Laterally, near the anterior part of scp, long cirri *A* and finger-like clava are located. However, it should be noted that in *Pse. clavatus* the clava is club-shaped, instead of finger-like, and that cephalic papillae are reduced. In *Pse. megacephalus*, the cephalic papillae are mushroom-shaped and additional papilliform projections between the external buccal cirri and cirri *A* are observed.

Spines on legs I are most often absent, although they are present in, for example, *Pse. alberti* Dastych, 1987. Papillae are always present on legs IV. A dentate collar on legs IV is usually absent, except in *Pse. alberti*, where one or two teeth are present in a dentate collar on legs IV. Spurs on claws are sharp, differing in size and directed downwards. They are present only on internal claws, but in some species they can also be absent (e.g. see: *Pse. yunnanensis* Wang, 2009 and *Pse. pilatoi* Li, 2007).

Genetic and morphological differences between examined *Pseudechiniscus* species

Based on our observations, main morphological differences between the examined species can be found on the ventral side of the body. Ventral sculpture is clearly unique, not only in all examined species but can also differ between the sexes of the same species. The other morphological and morphometric differences that we find, are more enigmatic and less obvious than the ventral sculpture. They are mostly related to the size of the dorsal granulation, the presence or absence of striae between the dorsal granules, the presence or absence of spines on legs I the size of the head appendages or claws. Morphological differences between the studied species are consistent with the significant differences in *COI* (22.1% on average) and ITS2 (26.4% on average) nucleotide sequences obtained in this study. In addition to the redescription of the nominal species, the morphological and genetic differences allow us to distinguish five new species in the genus *Pseudechiniscus* (which will be described in a subsequent work).

Taxonomic account

Phylum: Tardigrada Doyère, 1840
Class: Heterotardigrada Marcus, 1927
Order: Echiniscoidea Richters, 1926
Family: Echiniscidae Thulin, 1928
Subfamily Pseudechiniscinae Guil, Jørgensen & Kristensen, 2019
Tribe: Pseudechiniscini Guil, Jørgensen & Kristensen, 2019
Genus: *Pseudechiniscus* Thulin, 1911

Pseudechiniscus suillus (Ehrenberg, 1853) *sensu stricto*

(Table 4, Figs 3–5)

Material examined: Forty-five females (neotype and 44 neoparatypes) mounted on microscope slides in Hoyer's medium, ten females prepared for SEM and 11 females prepared for barcoding (exoskeletons mounted in Hoyer's medium as vouchers).

Redescription: animals (measurements and statistics in Table 4)

Females: Body (Fig. 4) yellow-orange in living specimens (transparent after mounting), eyes black after mounting. Apart from the head appendages [cirri *interni* and *externi* and spherical or slightly elongated cephalic papillae (secondary clava)], only lateral cirrus *A* present [with finger-like clava near the base (primary clava)] (Fig. 4A, B). Cephalic papillae smaller than primary clava.

Dorsal plates with small hemispherical granules/upper ends of cuticular pillars (dots in LM) 0.3–0.7 μm in diameter, densely (spaces between granules 0.3–1.1 μm) and uniformly distributed and not joined by striae (Fig. 5B). Granules/upper ends of cuticular pillars are slightly larger in the centre of the plates.

Dorsal plates typical for the genus *Pseudechiniscus* [single cephalic plate (cp), neck plate (np), scapular plate (scp), median plates (m1, m2, m3), paired segmental plates I and II (s1, s2), pseudosegmental plate (psp) and the caudal plate (cap), see 'Dorsal and ventral plates and sculpture' above] well developed. The cp facetted (with W-shaped pattern) divided into five parts (Fig. 4A, empty arrowhead). The scp divided by a transversal fold, which forms a long, narrow stripe in the posterior part of the plate. This narrow stripe is often divided by three longitudinal folds, resulting in four plate parts/subplates (Fig. 4A, B). Besides, the entire scp is divided by a median longitudinal fold into two parts (Fig. 4A, B, empty arrow). Additionally, lateral portions of the scp appear to be detached from the dorsal plate, forming small plate-like structures separated from the scp by a thin, bright stripe (Fig. 4A). Plates m1 and m2 are divided in two portions by a transverse fold; plate m3 is undivided (Fig. 4A, B, filled indented arrowheads). Laterally to the median plates, lateral intersegmental plates (lip) are present. On plates s1 and s2, darker stripes (folds in SEM) are also visible (Fig. 4A, filled arrow). The psp is divided by a longitudinal fold. Posterior margin of psp is straight, i.e. without projecting teeth or spines (Fig. 4A, B, empty indented arrowheads). The cap is concave with two Y-shaped bifurcated ridges (Fig. 4A, B, filled arrowhead). Ventral cuticle with tiny granulation (formed by dense granules/upper ends of cuticular pillars, 0.2–0.4 μm) forming a unique pattern (Figs 3, 4C, D, 5C). Ventral patches of granulation present, but most of them poorly marked and visible sometimes as a smooth areas almost without granulation (if granulation is present it is 0.3–0.5 μm in diameter, spaces between granules 0.2–0.3 μm), with configuration PG:I-II-III-IV-VI-VIII$_g$ (Figs 3, 4C,

Table 4. Measurements (in µm) and *sp* values of selected morphological structures of females of the neotype population of *Pseudechiniscus suillus s.s.* (Ehrenberg, 1853) mounted in Hoyer's medium [*N*, number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD, standard deviation; ?, trait oriented unsuitably for measurement; *sp*, ratio of the length of a given structure to the length of the scp expressed as a percentage (*sp* = length of the structure × 100/scp length)]

CHARACTER	*N*	RANGE						MEAN		SD		Neotype	
		µm			*sp*			µm	*sp*	µm	*sp*	µm	*sp*
Body length	15	142	–	186	*719*	–	*832*	164	*787*	13	*33*	169	*809*
Scapular plate length	15	18.8	–	23.5		–		20.9	–	1.5	–	20.9	–
Head appendages lengths													
Cirrus *internus*	13	8.7	–	11.1	*44.0*	–	*49.6*	9.6	*46.7*	0.8	*1.7*	9.3	*44.4*
Cephalic papilla	15	4.0	–	5.3	*19.1*	–	*24.3*	4.6	*22.1*	0.4	*1.6*	4.8	*22.9*
Cirrus *externus*	14	12.0	–	16.8	*62.1*	–	*75.0*	14.0	*67.1*	1.3	*4.2*	13.0	*62.1*
Clava	14	4.1	–	5.6	*20.9*	–	*26.8*	4.8	*22.9*	0.5	*1.7*	5.6	*26.8*
Cirrus *A*	15	28.4	–	34.4	*134.9*	–	*156.9*	30.5	*146.6*	1.9	*6.1*	30.0	*143.3*
Cirrus *A*/Body length ratio	15	17%	–	21%		–		19%	–	1%	–	18%	–
Cirrus *int*/*ext* length ratio	13	64%	–	76%		–		69%	–	4%	–	72%	–
Papilla on leg IV length	13	3.3	–	4.3	*14.7*	–	*18.4*	3.7	*17.5*	0.3	*1.0*	3.7	*17.5*
Claw 1 heights													
Branch	14	6.3	–	7.8	*30.6*	–	*35.8*	7.0	*33.7*	0.5	*1.3*	7.3	*34.9*
Spur	9	1.3	–	2.0	*6.9*	–	*9.3*	1.6	*7.7*	0.2	*0.7*	1.6	*7.6*
Spur/branch height ratio	9	21%	–	27%		–		23%	–	2%	–	–	–
Claw 2 heights													
Branch	13	5.9	–	7.0	*28.9*	–	*33.7*	6.5	*31.7*	0.4	*1.2*	6.9	*33.0*
Spur	9	1.3	–	1.8	*6.4*	–	*8.3*	1.5	*7.4*	0.2	*0.6*	1.5	*7.2*
Spur/branch height ratio	9	21%	–	26%		–		23%	–	2%	–	–	–
Claw 3 heights													
Branch	13	5.8	–	7.1	*30.7*	–	*33.7*	6.6	*32.0*	0.4	*0.9*	6.8	*32.5*
Spur	9	1.4	–	1.7	*6.9*	–	*8.3*	1.6	*7.5*	0.1	*0.4*	1.5	*7.2*
Spur/branch height ratio	9	22%	–	25%		–		24%	–	1%	–	–	–
Claw 4 heights													
Branch	15	6.7	–	8.7	*34.4*	–	*38.8*	7.6	*36.7*	0.6	*1.3*	7.5	*35.8*
Spur	12	1.5	–	2.0	*7.4*	–	*9.2*	1.7	*8.2*	0.1	*0.4*	1.6	*7.6*
Spur/branch height ratio	12	21%	–	24%		–		22%	–	1%	–	–	–

D). The female gonopore with the typical six-petal rosette (Fig. 4C, D, asterisks).

The outer cuticle on legs I–III has round patches of granulation (with larger granules but sparser in the centre and smaller and denser in peripheral parts); on legs IV, uniform wide stripes of granulation (slightly larger in the centre of these stripes) (Fig. 5D–F). Triangular spine on leg I absent, instead a small papilla-like structure present, but very hardly visible under LM (Fig. 5A). Dentate collar on leg IV absent. A finger-like papilla on leg IV present (Fig. 5E, filled arrow). External claws of all legs smooth, internal with spurs directed downwards (Fig. 5F).

Males: Unknown.

Remarks: The type material of *Pse. suillus* probably does not exist and the precise type locality of *Pse. suillus* is unknown. The species was described from the Monte Rosa massif, which is located between Switzerland and Italy. We examined more than 100 samples from this region and found a large population of individuals there that correspond well with the original description of *Pse. suillus*. The redescription of this species is important, because *Pse. suillus* is the nominal species for the *suillus–facettalis* complex and the type species of the genus *Pseudechiniscus*. In the last 165 years, *Pse. suillus* was reported by many authors throughout the world, but many characters in these records do not correspond to the original description and some recorded *Pse. suillus* specimens differ from each other, which strongly suggests that they belong to separate taxa. In this situation, the correct morphology and the distribution of the nominal *Pse. suillus* were unknown. Taking all these problems into consideration, we decided that the

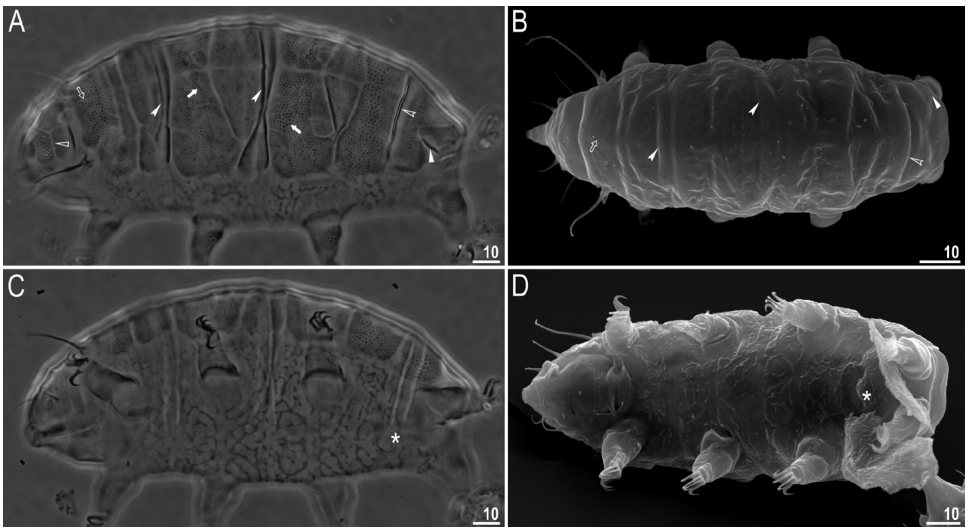

Figure 4. *Pseudechiniscus suillus s.s.*, female. A, B, dorsal image of the entire animal: empty arrowhead indicates the W-shaped pattern on the cephalic plate (cp); empty arrow indicates a median longitudinal fold dividing scapular plate (scp) into two parts; filled indented arrowheads indicate transverse folds dividing m1 and m2 in two portions; filled arrows indicate stripes (folds) on plates s1 and s2; empty indented arrowheads indicate straight pseudosegmental plate (psp); filled arrowheads indicate notches on caudal plate (cap) (neotype, PCM and neoparatype, SEM respectively). C, D, the characteristic pattern on the ventral side of the body; asterisks indicate female gonopore (neotype, PCM and neoparatype, SEM, respectively). Scale bars in micrometres (μm).

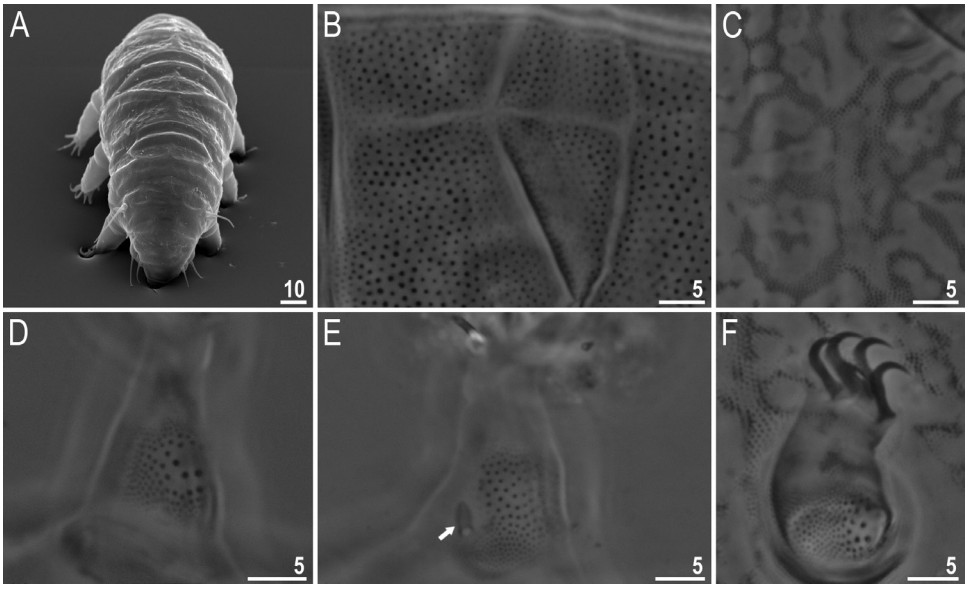

Figure 5. *Pseudechiniscus suillus s.s.*, female. A, front view of the individual (neoparatype, SEM). B, focus on dorsal plates s1 and s2, and m3 with visible upper ends of cuticular pillars (neotype, PCM). C, focus on characteristic pattern on the ventral side of the body above 3rd pair of legs (neoparatype, PCM). D, granulation on leg III (neotype, PCM). E, granulation and papillae (arrow) on leg IV (neotype, PCM). F, claws of leg III (neoparatype, PCM). Scale bars in micrometres (μm).

best solution is to designate a neotype population, a neotype locality and redescribe this species based on specimens found in the present study in the Monte Rosa. Determination of unambiguous morphological characters of *Pse. suillus s.s.* will facilitate further studies on the genus *Pseudechiniscus*, as well as correct identification and description of new taxa (see also Discussion below). Moreover, our specimens are

genetically similar to *Pse.* aff. *facettalis* from Italy (FJ435811–2) and Portugal (JX683830–1, MK804898) (for more details see Discussion below).

DNA sequences: We obtained good quality sequences for the analysed molecular markers:

– *COI* sequence (GenBank: MN528467), 696 bp long.
– ITS2 sequence (GenBank: MN537863), 457 bp long.

Neotype locality: 45°52'21"N, 07°51'52"E, 2858 m a.s.l.; Italy, Aosta Valley Province, Pennine Alps, Monte Rosa mountain massif, near Passo dei Salati, lichen on rock, 22 August 2019, coll. Tomasz Bartylak and Konrad Drygalski.

Type depositories: Neotype: slide IT.2 28/3 and 36 neoparatypes (slides: IT.2 28/*, where the asterisk can be substituted by any of the following numbers: 2, 3, 6, 8, 14, 16, 17, 19, 2/S, 9/S, 10/S, 13/S, 15/S, 16/S, 17/S, 19/S, 20/S, 23/S) are deposited at the Department of Animal Taxonomy and Ecology, Institute of Environmental Biology, Adam Mickiewicz University in Poznań, Uniwersytetu Poznańskiego 6, 61–614 Poznań, Poland; 12 neoparatypes (slides IT.2 28/7, IT.2 28/15, IT.2 28/18, IT.2 28/8/S) are deposited at the collection of Binda and Pilato, Museum of the Department of Animal Biology 'Marcello La Greca', University of Catania, Italy.

*Morphological differential diagnosis**

*Only measurements of adult females are used in differential diagnosis.

Pseudechiniscus suillus s.s. is the nominal species of the *suillus–facettalis* complex and differs specifically from:

1. ***Pse. beasleyi,*** known only from China (Li *et al.*, 2007), by: scp not divided in anterior part (scp divided into four parts in *Pse. beasleyi*), smaller granules of dorsal sculpture (0.3–0.7 μm in *Pse. suillus s.s.* vs. up to 1.6 μm in *Pse. beasleyi*), a different claw height pattern (claws II and III shortest and IV longest in *Pse. suillus s.s.* vs. claws I and II shortest and III and IV longest in *Pse. beasleyi*) and by shorter claws on all legs (5.8–8.7 μm in *Pse. suillus s.s.* vs. 9.1–13.1 μm in *Pse. beasleyi*).
2. ***Pse. clavatus*,** known only from Spain (Mihelčič, 1955), by: a different shape of clava (finger like in *Pse. suillus s.s.* vs. club-shaped in *Pse. clavatus*) and typically developed cephalic papillae (reduced in *Pse. clavatus*).
3. ***Pse. facettalis*,** known from distant localities throughout the world (McInnes, 1994). Based on present study, an inaccurate description of this species makes it impossible to differentiate this taxon from the nominal *Pse. suillus s.s.* (see also Discussion below).
4. ***Pse. jiroveci*,** known from China (type locality), South Africa and Tanzania (McInnes, 1994). Based on the present study, an inaccurate description of this species makes it impossible to differentiate this taxon from the nominal *Pse. suillus s.s.* (see also Discussion below).
5. ***Pse. juanitae*,** known from Austria, Brazil (type locality), Italy and Galapagos Islands (McInnes, 1994, but see also comments in Pilato & Lisi, 2006). Based on present study, an inaccurate description of this species makes it impossible to differentiate this taxon from the nominal *Pse. suillus s.s.* (see also Discussion below).
6. ***Pse. megacephalus*,** known only from Austria (type locality) and Turkey (McInnes, 1994), by: a different shape of the cephalic papilla (finger-like in *Pse. suillus s.s.* vs. mushroom-like in *Pse. megacephalus*), and the absence of a papilliform projection between the external buccal cirri and cirri *A*.
7. ***Pse. xiai*,** known only from China (Wang *et al.*, 2018), by: a different pattern of sculpturing on the ventral cuticle (Figs 3, 4C, D for *Pse. suillus s.s.* vs. figs 1B, F, 2E in Wang *et al.* (2018) for *Pse. xiai*), a longer cephalic papilla (4.0–5.3 μm in *Pse. suillus s.s.* vs. 1.4–3.9 μm in *Pse. xiai*), and by a higher cirrus *A*/body length ratio (17–21% in *Pse. suillus s.s.* vs. 13–16% in *Pse. xiai*).

Genotypic differential diagnosis

The ranges of genetic distances between *Pse. suillus s.s.* and species of the genus *Pseudechiniscus*, for which DNA sequences are available in GenBank, are as follows:

- *COI*: 3.7–28.2% (19.2% on average), with the most similar being *Pse.* aff. *facettalis* (JX683831, Vicente *et al.*, 2013a) and the least similar being *Pseudechiniscus* sp. (KJ857008, Velasco-Castrillón *et al.*, 2015) and *Pseudechiniscus* sp. 5 (MN528471, present study).
- ITS2: 6.8–39.3% (25.6% on average), with the most similar being *Pseudechiniscus* sp. 5 (MN537867, present study) and the least similar being *Pseudechiniscus* sp. 3 (MN537864, present study).

PHYLOGENETIC POSITION OF *PSEUDECHINISCUS SUILLUS SENSU STRICTO* BASED ON *COI*, ITS2 AND COMBINED *COI*+ITS2 SEQUENCES

The analysis of *Pseudechiniscus COI* sequences deposited in GenBank and obtained in this study (Fig. 6) clearly distinguishes species belonging to the

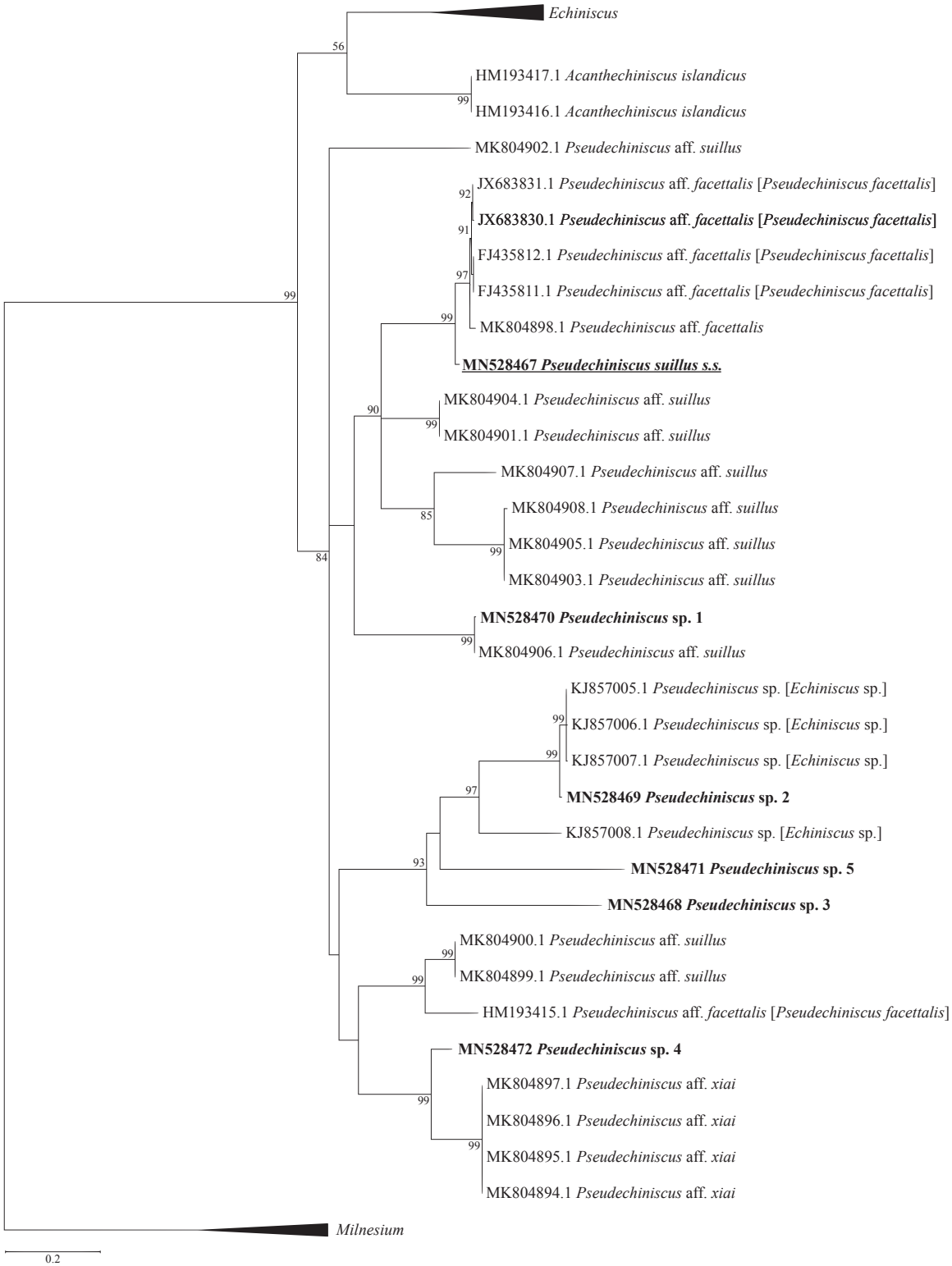

Figure 6. Molecular phylogenetic analysis based on *COI* sequences and performed by maximum likelihood method. MEGA 7 was used to prepare the tree rooted on the genus *Milnesium*. Support values (above 50%) for the tree were assessed with 1000 bootstraps and are marked at the nodes. Species names in square brackets are GenBank labels that are incorrect species identifications, uncertain identifications or invalid names (correct identifications are provided before square brackets). New sequences are marked with bold font.

genus *Pseudechiniscus* from those belonging to other genera. Based on the phylogenetic tree shown in Figure 6, we can conclude that sequences obtained from Antarctica and deposited in GenBank as *Echiniscus* sp. (KJ857005–8) belong to species representing the genus *Pseudechiniscus*, whereas the sequence from Chile labelled in GenBank, as *Pse. novaezeelandiae* (HM193418) belongs to a species from the genus *Echiniscus* (sequence located within the *Echiniscus* clade).

The *COI* phylogenetic tree (Fig. 6) supports the presence of three distinguished groups within the genus *Pseudechiniscus*. The first contains only *Pse.* aff. *suillus* (MK804902) from Mongolia. The second, the other sequences from Mongolia (MK804901, MK804903–8), *Pseudechiniscus* sp. 1, *Pse. suillus s.s.* (MN528467), both from Italy, as well as *Pse.* aff. *facettalis* from Italy (FJ435811–2) and Portugal (JX683830–1 and MK804898). Based on the phylogenetic tree, we can conclude that *Pse.* aff. *suillus* from Mongolia (MK804903, MK804905, MK804908) is related to other *Pse.* aff. *suillus* (MK804907), also from Mongolia, whereas two other Mongolian taxa also named *Pse.* aff. *suillus* (MK804901, MK804904) represent another species. The phylogenetic position of the redescribed nominal *Pse. suillus* and low genetic distances in *COI* (3.7–4.1%) indicate that this species is closely related to *Pse.* aff. *facettalis* from Italy (FJ435811–2) and Portugal (JX683830–1, MK804898), but the lack of ITS2 sequences and morphological characteristics for specimens from Portugal makes it (currently) impossible to answer the question whether these populations represent *Pse. suillus s.s.* or a different species. The third group contains *Pseudechiniscus* sp. from Antarctica (KJ857005–8), *Pseudechiniscus* sp. 2 from Antarctica, *Pseudechiniscus* sp. 3 from Madagascar, *Pseudechiniscus* sp. 4 and 5 from Norway, *Pse.* aff. *xiai* from Slovakia (MK804894–7), *Pse.* aff. *suillus* from Italy (MK804899–900) and *Pse.* aff. *facettalis* (HM193415) from Greenland. It is clear that all *Pse.* aff. *xiai* sequences from Slovakia (MK804894–7) belong to the same species, which is related to *Pseudechiniscus* sp. 4 from Norway. The sequences of *Pse.* aff. *suillus* (MK804899–900) from Italy are related to *Pse.* aff. *facettalis* (HM193415) from Greenland. Moreover, our analysis shows that the sequences from Antarctica (KJ857005–7) deposited in GenBank as *Echiniscus* sp. (Velasco-Castrillón *et al.*, 2015), most likely represent *Pseudechiniscus* sp. 2, which exhibits the same ventral pattern as the species earlier reported from Antarctica by Dastych (1984) as *Pse. suillus*. In the phylogenetic tree, these species are closest to another *Pseudechiniscus* species (also reported as *Echiniscus* sp. in GenBank) from Antarctica (KJ857008) and then to *Pseudechiniscus* sp. 5 from Norway, and to *Pseudechiniscus* sp. 3 from Madagascar.

The analysis of ITS2 sequences of *Pseudechiniscus* spp. obtained in this study (Fig. 7) allows us to distinguish two clades. The first one contains *Pse. suillus s.s.* and two species from Norway (*Pseudechiniscus* sp. 4 and *Pseudechiniscus* sp. 5) and the second one includes *Pseudechiniscus* sp. 1 from Italy, *Pseudechiniscus* sp. 2 from Antarctica and *Pseudechiniscus* sp. 3 from Madagascar.

The analysis of combined *COI* and ITS2 (Fig. 8) also allows us to distinguish the same two clades as in the case of ITS2 (see Fig. 7).

DISCUSSION

MORPHOLOGY

Species of the genus *Pseudechiniscus* are characterized by a uniform morphology. Lateral and dorsal appendages are absent or limited to short spines or lobes, situated mainly on the posterior margin of the psp. Almost all species with longer lateral filaments, previously attributed to the genus *Pseudechiniscus*, have recently been transferred to the newly described

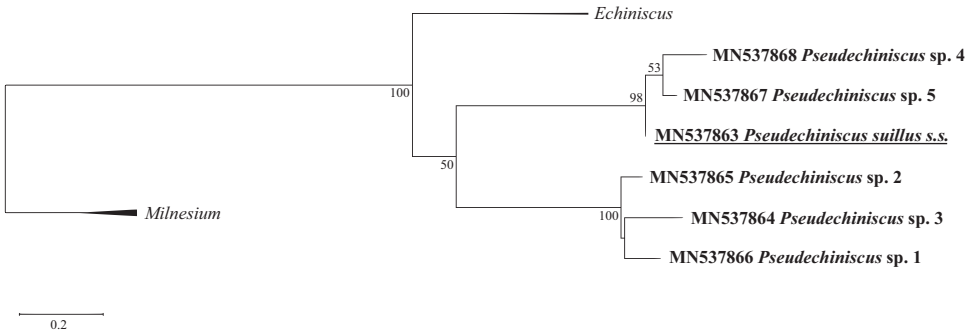

Figure 7. Molecular phylogenetic analysis based on ITS2 sequences and performed by maximum likelihood method. MEGA 7 was used to prepare the tree rooted on the genus *Milnesium*. Support values (above 50%) for the tree were assessed with 1000 bootstraps and are marked at the nodes. New sequences are marked with bold font.

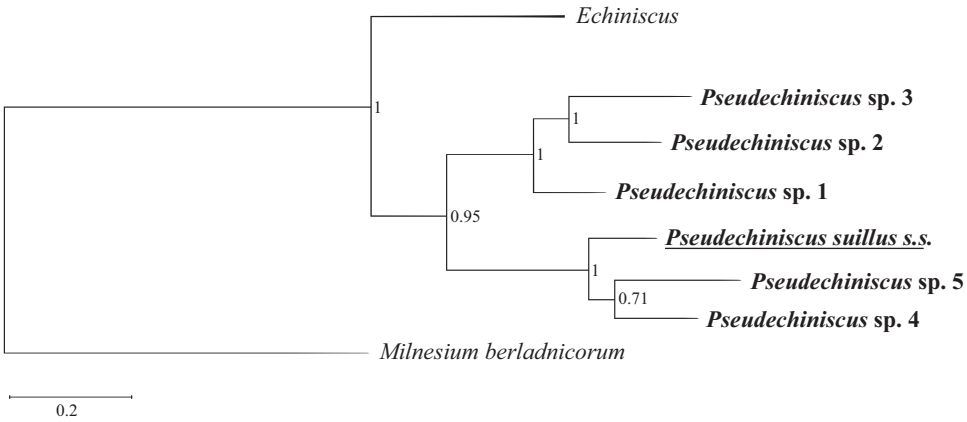

Figure 8. Molecular phylogenetic analysis performed based on combined *COI* and ITS2 sequences and performed by maximum likelihood method. FigTree was used to prepare the tree. Posterior probabilities are marked at the nodes. The tree was rooted with the *Mil. berladnicorum* combined sequences. New sequences are marked with bold font.

genus, *Acanthechiniscus*. The dorsal plates are covered in more or less developed granulation, formed by the upper ends of cuticular pillars localized under the epicuticle. In some species, these granules are additionally connected by thin striae (stripes positioned under the epicuticle), forming a delicate hexagonal structure. These striae can be clearly pronounced or thin and poorly visible (e.g. see: Fontoura *et al.*, 2010). In addition, all the dorsal plates are divided by variously developed folds of cuticle. The legs are long, with patches or stripes of granulation. The spine on leg I may be present or absent, but the papilla on leg IV is always present. Claws are typical for Echiniscidae, smooth or with basal spurs on internal claws. The dentate collar is almost always absent [except for *Pse. alberti* and an unverified taxon *Pse.* cf. *papillosus* reported by Beasley & Miller (2012)]. Some *Pseudechiniscus* species are dioecious, but in others only females were observed.

Pseudechiniscus suillus, the nominal species not only for the so-called *suillus* group, but also for the genus *Pseudechiniscus*, was described 165 years ago and according to current standards, the description is insufficient. Since then, different authors have interpreted the morphology of this taxon in different ways. Due to this approach, this species has been considered to be variable and widely distributed (e.g. see: Marcus, 1936; Ramazzotti & Maucci, 1983; McInnes, 1994; Kaczmarek *et al.*, 2014, 2015, 2016; McInnes *et al.*, 2017). The situation became even more complicated following the insufficient descriptions of species morphologically similar to *Pse. suillus*, i.e. *Pse. facettalis*, *Pse. jiroveci* and *Pse. juanitae*. All these species were described based on enigmatic characters, such as faceted plates, granulation size or the presence of additional plates. From the beginning these taxa were confused, mainly due to morphological characters of *Pse. suillus* being unclear.

Pseudechiniscus beasleyi has recently been described and, although its description is much more thorough, there is still some ambiguity, which does not allow for a precise discrimination of this species from other members of the *suillus* group. It should also be noted that some of the described taxa were later synonymized with other members of the *suillus–facettalis* complex (e.g. see: Marcus, 1936; Ramazzotti & Maucci, 1983; Degma *et al.*, 2009–18). Even though the morphological characters of all members of the *suillus–facettalis* complex were unclear and enigmatic, *Ech. mutabilis* and *Pse. suillus papilalta* Rahm, 1931 were synonymized with *Pse. suillus* (Marcus, 1936), *Pse. suillus franciscae* with *Pse. juanitae*, and *Pse. pseudoconifer facettalis* Maucci, 1954 with *Pse. facettalis* (Ramazzotti & Maucci, 1983). The situation was further complicated by the fact that some authors did not mention the sex of the analysed specimens or described species using only males. Furthermore, *Pse. jiroveci* was described using only larvae (Bartoš, 1963). In older descriptions, the characterization of crucial features, such as claw spurs, details of dorsal plate morphology and sculpture or ventral sculpture pattern, as well as morphometrics, are missing. Up to now, only one species – *Pse. juanitae* – has been partially redescribed on the basis of non-type material (Pilato & Lisi, 2006).

Here, we have shown that one of the most important characters in the *suillus–facettalis* complex (and possibly also in the entire genus *Pseudechiniscus*) may be the structure of the ventral sculpture, which is unique at the species level (and was also supported by genetic analyses). The ventral pattern is highly complex and conservative, which has also been reported by Pilato *et al.* (2001) for *Pse. spinerectus* Pilato *et al.*, 2001). However, it can differ greatly between males and females of the same species. The ventral pattern is

well visible only when high-quality PCM or Nomarski contrasts are applied. This could explain why this character has been ignored for so long in the taxonomy of this genus. Even if the sculpture had been observed, researchers ignored it as being an allegedly variable character that was not important from the taxonomic point of view (probably due to considering different *Pseudechiniscus* species as *Pse. suillus*).

The ventral sculpture has been reported, for example, in *Pse. asper* Abe *et al.*, 1998 (from Japan), *Pse. brevimontanus* Kendall-Fite & Nelson, 1996 (from the USA), *Pse. gullii* Pilato & Lisi, 2006 (from Mexico), *Pse. jiroveci* (from Africa), *Pse. jubatus* Biserov, 1990 (from Russia), *Pse. nataliae* (from Russia), *Pse. santomensis* (from São Tomé Island), *Pse. spinerectus* (from Ecuador), *Pse. suillus* (now *Pseudechiniscus* sp. 2) (from Antarctica) or *Pse. xiai* (from China) (Binda, 1984; Dastych, 1984; Biserov, 1986, 1990; Kendall-Fite & Nelson, 1996; Abe *et al.*, 1998; Pilato *et al.*, 2001; Pilato & Lisi, 2006; Fontoura *et al.*, 2010; Wang *et al.*, 2018). However, in most species descriptions the ventral sculpture has not been presented (described and/or drawn) in detail (e.g. see: *Pse. gullii, Pse. jiroveci* or *Pse. jubatus*). In others, the ventral sculpture has been described and/or drawn only in males (e.g. see: *Pse. asper* or *Pse. nataliae*). The only species with the sculpture well described and illustrated are *Pse. santomensis* and *Pse. xiai* (Fontoura *et al.*, 2010; Wang *et al.*, 2018).

The majority of the species mentioned above (except for *Pse. jiroveci*, *Pse. xiai* and *Pseudechiniscus* sp. 2) do not belong to the *suillus–facettalis* complex, being characterized by the presence of dorsal spines or lobes. The observations of previous authors, and coming from the present study, suggest that the ventral sculpture is frequently present in *Pseudechiniscus* species and is significant from the taxonomical point of view. What is also important, the ventral sculpture can appear different in males and females of the same species. For example, *Pse. asper* was described with a single male individual and it is difficult to state whether this male does not actually belong to one of the formerly designated species, which were described using females. The open question is whether this character differs between life stages (larvae, juveniles and adults).

The next problematic character is the presence or absence of false divisions of the dorsal plates (forming separate plate parts/subplates). These false divisions are formed by cuticular folds, visible as bright lines under PCM or prominent ridges under SEM. As noted above, the dorsal plates in *Pseudechiniscus* are soft and elastic. This cuticle 'softness' in *Pseudechiniscus* species has confused the interpretation of the dorsal plate structure, a fact that has been previously mentioned by, for example, Kristensen (1987) in his comprehensive and highly detailed revision of the Echiniscidae. Furthermore, Kristensen (1987) stated that lip's are present only in more 'evolutionarily advanced' *Pseudechiniscus* taxa with lateral filaments (these taxa are now attributed to the genus *Acanthechiniscus* or grouped in the *Pse. conifer* complex). However, lip's are also present in species of the *suillus* group, which indicates that they are probably present in all taxa attributed to the *Pseudechiniscus* evolutionary lineage. Pilato *et al.* (1991) stated that the false divisions of plates (folds) '…can also disappear under pressure from the cover glass…', which was confirmed in our study. Consequently, it is difficult to determine which species of the *suillus–facettalis* complex and the genus *Pseudechiniscus* in general have such plate parts/subplates and what their arrangement is. At present, we suggest using this character in differential diagnoses with caution. We also suggest preparing two types of microscope slides with *Pseudechiniscus* specimens, i.e. (1) with a thick layer of mounting medium that allows the observation of details of the dorsal plates and (2) thin ones that are better for a general study of all the other morphological characters. For the correct identification of folds and plate parts/subplates, SEM photographs are useful, but not obligatory.

TAXONOMY

Until now, six genera have been designated for species earlier attributed to the genus *Pseudechiniscus*: *Mopsechiniscus* du Bois-Reymond Marcus, 1944, *Cornechiniscus* Maucci & Ramazzotti, 1981, *Antechiniscus* Kristensen, 1987, *Proechiniscus* Kristensen, 1987, *Multipseudechiniscus* Schulte & Miller, 2012 and *Acanthechiniscus*. Members of the genus *Mopsechiniscus* are characterized by the absence of external and internal buccal cirri. In the genus *Cornechiniscus*, all species possess characteristic cirri *A* in the shape of cones. Species placed in *Antechiniscus*, *Proechiniscus* and *Multipseudechiniscus* were excluded from *Pseudechiniscus* mainly based on specific arrangements of the dorsal plates. Finally, *Acanthechiniscus* was established for ex-*Pseudechiniscus* taxa possessing lateral (in positions *B, C, D, E*) and/or dorsal filaments/spines and a dentate collar on legs IV. Consequently, almost all species with long lateral appendages, spines on legs I and a dentate collars on legs IV have been excluded from *Pseudechiniscus*, with the exception of *Pse. alberti* (spine on leg I and dentate collar on leg IV present), *Pse. bispinosus* [(long lateral spines *C* present), *Pse. n. novaezeelandiae* (spine on leg I present according to Ramazzotti & Maucci (1983), but on the photos of this species included in Pilato *et al.* (2005) the spine is not visible, suggesting that the spine is probably absent (this needs to be confirmed in further studies and a redescription of this taxon)], *Pse. pulcher*

(long lateral filaments *E* present), *Pse. scorteccii* Franceschi, 1952 (spine on leg I present) and *Pse. transsylvanicus* (lateral filaments *C* present). The nominal species of *Pseudechiniscus* is characterized by the absence of spine on leg I (instead a small papilla-like structure present, but very hardly visible under LM), absence of dentate collar on leg IV and the lack of dorsal or lateral trunk appendages. This suggests that the attribution of species characterized by a different set of characters to *Pseudechiniscus* should be considered only as temporary. This is especially true for the species listed above, characterized by the the presence of lateral filaments and a dentate collars on leg IV. A separate group of *Pseudechiniscus* species, characterized by the presence of short dorsal and/or lateral spines and/or lobes (*Pse. conifer* group), should be probably attributed to a separate genus or genera, but this needs confirmation based on detailed morphological and genetic studies.

Two more problematic species with unique characters are *Pse. clavatus*, with a club-shaped clava, and *Pse. megacephalus*, with mushroom-shaped cephalic papillae and an additional papilliform projection between the external buccal cirri and cirri *A*. If the presence of these characters is confirmed, then both taxa will need to be excluded not only from the *suillus* group, but probably also from the genus *Pseudechiniscus*.

Another three species of the *suillus* group, namely *Pse. facettalis, Pse. jiroveci* and *Pse. juanitae*, are problematic because of inadequate descriptions or doubtful characters that were used for the discrimination of these taxa. *Pseudechiniscus facettalis* was described mainly based on the presence of faceting on the cp and cap (Petersen, 1951), but, as stated above, such faceting is typical in many *Pseudechiniscus* taxa and is mostly caused by the 'softness' of the dorsal cuticle. Moreover, as shown above, some specimens from Italy and Portugal attributed to *Pse.* aff. *facettalis* by some authors (Guil & Giribet, 2012; Vicente *et al.*, 2013a; Cesari *et al.*, 2019) are genetically similar to *Pse. suillus s.s.*, which has been confirmed by the *COI* analyses in the present study. However, sequences deposited in GenBank were obtained from specimens collected far away from the type locality of *Pse. facettalis* and it is possible that they were misidentifications. Moreover, the ITS2 sequences for these specimens are unknown and an indisputable attribution of them to *Pse. suillus* is not possible. *Pseudechiniscus jiroveci* was described based on four larvae, and it is characterized mainly by the presence of four plate parts/subplates, resulting from the division of the scp (Bartoš, 1963). However, this character is also frequent in other species of *Pseudechiniscus*, although it might have been overlooked or ignored by other authors due to problems with interpretation of false divisions of plates in the genus. *Pseudechiniscus juanitae*, differs from *Pse. suillus* by the presence of larger granules on the dorsal plates (de Barros, 1939). It was partially redescribed by Pilato & Lisi (2006), but the exact dimensions of these granules (not ascribed to *Pse. suillus* prior to the redescription herein) were not reported. The same applies to the original description of *Pse. juanitae*. In addition, in all of these taxa, the ventral sculpture was not examined. Therefore, we suggest considering these taxa to be *species dubia*, all requiring a redescription based on the type material or new specimens from the type localities. Nevertheless, considering that all three taxa were described from distant localities, including South America, Greenland and Asia, it is highly probable that they belong to separate taxa within the *suillus–facettalis* complex. Moreover, *Ech. mutabilis* and *Pse. suillus papilalta* were synonymized with *Pse. suillus* (Marcus, 1936), but according to our data (mainly concerning the ventral sculpture), these species should also be considered as *species dubia*, although, potentially after a redescription, they could become accepted species of the *suillus–facettalis* complex.

The performed genetic analysis demonstrated that the application of molecular methods in modern taxonomy of *Pseudechiniscus* species is important, especially because of the high morphological intraspecific variability reported in Echiniscidae (e.g. see: Pilato, 1972; Binda & Guglielmino, 1982; Ramazzotti & Maucci, 1983; Maucci, 1985; Binda & Pilato, 1994; Guil, 2008; Vicente *et al.*, 2013b; Gąsiorek *et al.*, 2017, 2018, 2019; Bartylak *et al.*, 2019). It is also known that the genetic distances between tardigrade species can be high e.g. see: Guil & Giribet, 2009; Bertolani *et al.*, 2011; Faurby *et al.*, 2011; Guidetti *et al.*, 2019; Morek *et al.*, 2019), which we confirmed for the genus *Pseudechiniscus* in the present study. Similarly high p-distances (up to 33.3%) were also reported in the genus *Pseudechiniscus*, even for the specimens from the same locality by Cesari *et al.* (2020). Concerning the fact that we have not observed high intraspecific variability among the studied species, we suspect that this variation is a result of interspecific differences. The significant differences in *COI* (22.1% on average) and ITS2 (26.38% on average) sequences and morphology of ventral sculpture obtained in our study allowed us to distinguish new species in the genus *Pseudechiniscus* (which will be described in a subsequent work). However, the high number of gaps existing in *Pseudechiniscus* ITS2 sequences, which were partially excluded from the analysis, should be also mentioned.

Recently, Cesari *et al.* (2020) showed that two morphologically distinct groups in the genus *Pseudechiniscus*, the *Pse. novaezeealandiae* group (characterized by an elongated cephalic papilla) and the *Pse. suillus–facettalis* group (characterized by a dome-like cephalic papilla), are genetically well-supported

lineages. Curiously, a *COI* sequence deposited in GenBank as *Pse. novaezeelandiae* (HM193418) was not used by Cesari *et al.* (2020) and it was not discussed as to why this particular sequence was excluded from their analysis. Based on the phylogenetic analysis, we suspect that Cesari *et al.* (2020), similarly to our own findings, concluded that this sequence belongs to an undefined species of the genus *Echiniscus*. Our phylogenetic analysis of *COI* sequences highlights another problematic sequence deposited in GenBank as *Pse. facettalis* (HM193415), which does not cluster with other *Pse.* aff. *facettalis* sequences (FJ435811–2, JX683830–1 and MK804898) and probably belongs to another species of the *suillus–facettalis* complex.

The analysis of *Pseudechiniscus COI* sequences deposited in GenBank allows us to conclude that redescribed nominal *Pse. suillus s.s.* is at least closely related, if not the same, species as *Pse.* aff. *facettalis* from Italy (FJ435811–2) and Portugal (JX683830–1, MK804898). However, as stated above, the lack of available ITS2 sequences from the genus *Pseudechiniscus* prevents a confident conclusion. Instead, the phylogenetic analysis of ITS2 sequences and combined *COI*+ITS2 sequences allows us to distinguish different clades that are identical in both analyses (see Figs 7, 8). Therefore, it can be concluded that ITS2 sequences played a key role in obtaining the presented results. Different positions of *Pse. suillus s.s.* on these phylogenetic trees may be a result of different evolution rates of these molecular markers in Tardigrada. However, a small number of *Pseudechiniscus* sequences deposited in GenBank does not allow for more advanced analyses at this moment.

Considering the data, misunderstandings, misinterpretations and insufficient species descriptions, the majority of *Pseudechiniscus* taxa need to be redescribed using modern taxonomy and, if possible, also genetic analysis. In the *Pse. suillus* group, only two species have complete (redescribed *Pse. suillus*) or almost complete (*Pse. xiai*) descriptions. Two others, *Pse. clavatus* and *Pse. megacephalus*, require redescriptions because they possibly belong to a different genus. Additionally, five taxa (*Ech. mutabilis, Pse. facettalis*, *Pse. jiroveci*, *Pse. juanitae* and *Pse. suillus papilalta*), although definitively belonging to the *suillus–facettalis* complex, also require redescriptions due to insufficient descriptions in the past.

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