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Taxonomic insights into the phylogeny of *Bacillus badius* and proposal for its reclassification to the genus *Pseudobacillus* as *Pseudobacillus badius* comb. nov. and reclassification of *Bacillus wudalianchiensis* Liu et al., 2017 as *Pseudobacillus wudalianchiensis* comb. nov.

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ABSTRACT

The species *Bacillus badius* is one of the oldest members of the genus *Bacillus* isolated from faeces of children and was classified based on its ability to form endospores [8]. In 16S rRNA gene sequence and phylogenetic analysis, *Bacillus badius* DSM 23^T shared low similarity (93.0%) and distant relationship with *B. subtilis*, the type species of the genus *Bacillus* indicating that it does not belong to this genus. Additional strains of the species, *B. badius* DSM 5610, DSM 30822 and *B. encimensis* SGD-V-25 (which has been recently reclassified as a member of this species) were included in the study to consider intraspecies diversity. Detailed molecular phylogenetic and comparative genome analysis clearly showed that the strains of *B. badius* were consistently retrieved outside the cluster of *Bacillus sensu stricto* and also distantly related to the genera *Domibacillus* and *Quasibacillus*. Further, the data from biochemical reactions (inability to ferment most carbohydrates), polar lipids profile (presence of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an aminophosphoglycolipid) and fatty acids supported the molecular analysis. Thus the four *B. badius* strains; DSM 23^T, DSM 5610, DSM 30822 and SGD-V-25 displayed sufficient demarcating phenotypic characteristics that warrant their classification as members of a novel genus and single species, for which the name *Pseudobacillus badius* gen. nov. comb. nov. is proposed with *Pseudobacillus badius* DSM 23^T (= ATCC 14574^T) as the type strain. Additionally, based on our findings from phenotypic, chemotaxonomic and genotypic parameters, *Bacillus wudalianchiensis* DSM 100757^T was reclassified as *Pseudobacillus wudalianchiensis* comb. nov.

The taxonomy of the genus *Bacillus* is very heterogeneous and it is an accepted fact that many of its species require reclassification into novel or already existing genera. This is especially true for the older species classified by taxonomists before the advent of molecular phylogeny, solely on the basis of phenotypic criteria of Gram-staining-positive bacteria forming endospores with few biochemical test parameters. At that time chemotaxonomic criteria such as fatty acids, quinones and polar lipids did not form an integral part of species/genus descriptions. Some of the noted examples include *Bacillus insolitus*, *B. sphaericus*, *B. fusiformis*, *B. psychrophilus*, *B. globiformis*, *B. pasteurii*, *B. lautus*, *B. arvi*, *B. arenosi*, *B. neidei* etc. which were later reclassified into novel genera like *Psychrobacillus* [26], *Lysinibacillus* [1], *Sporosarcina* [17,62], *Paenibacillus* [5] and *Viridibacillus* [2], respectively. One of the oldest *Bacillus* species, *Bacillus badius* was first described by Batchelor [8] and rediscovered in 1952 by Saghafi and Appleman [47] who described its phenotypic properties in more detail and recommended its description in the first edition of Bergey's Manual of Systematic Bacteriology

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[13]. With 16S rRNA gene sequencing becoming part of phylogenetic analysis, studies by few researchers (Ash et al. [4], Farrow et al. [17,18]) made it clear that *B. badius* was only distantly related to *B. subtilis* (*Bacillus sensu stricto*). In fact, different treeing algorithms recovered *B. badius* DSM 23^T as a separate lineage between *Bacillus* groups 1 and 2. However lack of additional related strains and a coherent taxonomic framework for the genus *Bacillus* prevented any revisions in the taxonomy of this species. During our investigations of bacterial diversity of different marine and soil habitats, we have isolated and characterized several strains which showed close phylogenetic proximity to *B. badius* [58–60]. These strains seemed to share a distant relationship with *Bacillus sensu stricto* in trees constructed using different treeing algorithms. The branching pattern was stable and they were always retrieved outside of the genus *Bacillus* thus confirming the findings of earlier researchers and our own laboratory. To further introspect the taxonomy of *B. badius*, we analyzed four strains i.e., one type strain DSM 23^T, two reference strains DSM 5610, DSM 30822 and one type strain of *Bacillus encimensis* SGD-V-25 that was recently reclassified as a member of *B. badius* [58]. For the comparative phenotypic and genotypic characteristics, the type species of the genus *Domibacillus* [52] and *Quasibacillus* [58] were also taken into consideration. Using data from phenotypic and chemotaxonomic studies as well as whole genome sequencing we present evidence for description of a novel genus, *Pseudobacillus badius* gen. nov. comb. nov., with strain *B. badius* DSM 23^T as the type strain of the type species of the genus and the other two strains (DSM 5610 and DSM 30822) belonging to the same species. Moreover, one more recently described species i.e., *Bacillus wudalianchiensis* [31] was found to be retrieved in the clade of the novel genus based on 16S rRNA gene phylogenetic analysis and its classification as a separate species of this genus was also supported by phenotypic, chemotaxonomic and genomic parameters and thereby is reclassified as *Pseudobacillus wudalianchiensis* comb. nov.

The strains *B. badius* DSM 23^T, DSM 5610, DSM 30822, *B. wudalianchiensis* DSM 100757^T, *Domibacillus robiginosus* DSM 25058^T, *Quasibacillus thermotolerans* SgZ-8^T and *B. subtilis* DSM 10^T were obtained from DSMZ, Germany. The strain *B. encimensis* SGD-V-25 was obtained from a co-author of this manuscript (Dr Syed G. Dastager, CSIR-NCL, Pune, India). Morphological properties like size, shape and motility were studied according to standard protocols [53]. The Gram's reaction was determined using the HiMedia Gram staining kit according to the manufacturer's instructions. For scanning electron microscopy (SEM), the strain *B. badius* DSM 23^T was grown in 2 ml TSB overnight at 30 °C and cells were harvested in a microcentrifuge at 4000 r.p.m. at 4 °C for 10 min and further processed that involved washing, fixing and drying of cells. The harvested cells were washed thrice with PBS (pH 7.2) and spread evenly over polylysine coated cover slips. The cells were fixed using 2% (v/v) glutaraldehyde for 1.5 h at 4 °C and washed again with PBS to remove unadhered cells. A series of alcohol dehydration steps (at increasing concentrations of 30, 50, 70, 90 and 100% (v/v) were followed and finally the cells were layered with tertiary butanol (tert-butyl alcohol) for freeze drying. The samples were then viewed under a scanning electron microscope (Zeiss, Evo 40). Tests like catalase, oxidase (using N,N,N′,N′,-tetramethyl-*p*-phenylenediamine dihydrochloride impregnated discs), hydrolysis of casein, gelatin, starch and urea were determined as described by Smibert and Krieg [53]. The range and the optimum pH for the strains were determined using biological buffers as mentioned earlier [12]. MR-VP (Methyl Red-Voges Proskauer), indole, nitrate reduction and acid production were determined as described by Lányi [28] and tests such as utilization of carbohydrates, acid production and various enzymatic activities were done using API 20NE, API ZYM kits and OMNILOG GEN III system (Biolog) according to the manufacturer's instructions. The substrate oxidation pattern in GEN III system (Biolog) between the strains was depicted as heatmap and hierarchical clustering using RetroSpect2 software (Omnilog) as per the manufacturer's instructions.

For cellular fatty acid analysis the strains were grown on tryptic soy broth agar (TSBA) medium at 30 °C for 48 h and the fatty acid methyl ester analysis was performed by using the Sherlock Microbial Identification System (MIDI version 6.1, database RTSBA 6.0) as described previously (MIS operating manual version 6.1 [49,58]). Hierarchical clustering of strains based on cellular fatty acids was performed using fastcluster R script [15]. For chemotaxonomic analyses (polar lipids, quinones and whole cell diaminoacid) the strains *B. badius* DSM 23^T, DSM 5610, DSM 30822 and SGD-V-25 and *B. wudalianchiensis* DSM 100757^T were cultivated in TSB for 2 days in a rotary shaker (200 r.p.m.) at 30 °C. Cells were harvested by centrifugation in a R10A3 rotor (Hitachi) at 17,000 × *g*. Extraction of isoprenoid quinones and polar lipids were done as earlier described [10,37,58]. Two-dimensional TLC was run for detection and identification of polar lipids according to procedures described by Komagata and Suzuki [25] and Krishnamurthi et al. [26], respectively. The menaquinones were analyzed by reverse phase TLC as explained by Collins and Jones [14]. Whole cell diaminoacid analysis for detection of diaminopimelic acid (Dpm) isomers was done as per Protocol 1 of Schumann [50]. MALDI-TOF mass spectra measurements were carried out using an Ultraflex III instrument (Bruker Daltonik) operated on default settings as described previously [57]. Furthermore, riboprinting was undertaken using an automated Riboprinter (Hygiena) with *Eco*RI as the restriction enzyme in order to analyze the intraspecies diversity [3,51].

Genomic DNA was isolated according to the method described by Pitcher et al. [43]. The 16S rRNA gene was amplified by PCR using the universal primers 8-27F (5′ AGAGTTTG-ATCCTGGCTCAG 3′) and 1492R (5′ TACGGYTACCTTGTTACGACTT 3′). Amplification and purification of the product was done as described previously [42]. The amplified 16S rRNA gene was sequenced by the dideoxy chain terminator method using a Big Dye terminator kit followed by capillary electrophoresis on an ABI 3130xl Genetic Analyzer (Applied Biosystems). The primers used for sequencing were 685R (5′ TCTACGCATTTCACCGCTAC 3′), 533F (5′ GTGCCAGCMGCCGCG-GTAA 3′) and 1492R. The identification of phylogenetic neighbors was initially carried out using a program to check against the database of type strains of prokaryotes with validly published names in the EzTaxon server (http://www.ezbiocloud.net/eztaxon) [24]. The 16S rRNA gene sequences of closely related species with valid names were retrieved from the NCBI database. The final construction of tree was done using FastTree v 2.1.1 [44] and the tree was rendered in iTOL [30]. The *gyrB* gene and the amino acid sequences of *Pseudobacillus* and related strains were also taken into consideration for better resolution of the phylogenetic distance using MEGA6.0 [55].

Extraction of genomic DNA from strains *B. badius* DSM 30822, DSM 5610 and SGD-V-25 for whole genome sequencing (WGS) was done using the Genomic tip DNA extraction kit (Qiagen) according to the manufacturer's instructions after growing them in TSB at 30 °C for 2–3 days under shaking conditions in 500 ml culture broth. Library preparation was performed at Genotypic Technologies Genomics facility following NEXTFlex DNA library protocol outlined in NEXTFlex DNA sample preparation guide (Cat # 5140-02). The whole genome sequencing of the strains were completed as outlined earlier [60]. The de novo assembly of paired end reads for all the organisms were done using SPAdes v3.1 [7] and CLC Genomics Workbench software version 7.0.3 using default settings. The genomes were assembled with different parameters. The contigs thus obtained for all the organisms were scaffolded independently using SSPACE v2.0 [11] and the gaps were filled by GapFiller v1.10 [38]. The gap-filled scaffolds thus obtained, were broken into contigs where gaps were not filled. Functional

Table 1
Comparative phenotypic characterization of *Pseudobacillus badius* strains DSM 23^T (1), DSM 5610 (2), DSM 30822 (3), SGD-V-25 (4), *Pseudobacillus wudalianchiensis* DSM 100757^T (5), *Quasibacillus thermotolerans* SgZ-8^T (6), *Domibacillus robiginosus* DSM 25058^T (7), *B. subtilis* subsp. *subtilis* DSM 10^T (8). +, positive; –, negative; [+], weakly positive; R, rod; LR, large rod; SR, small rod; E, ellipsoidal; S, spherical; E-S, ellipsoidal to spherical; T, terminal; C, central; ST-T, Subterminal to terminal; C-PC, central to pericentral; C-ST, central to subterminal; C-PC-ST, central to pericentarl to subterminal.

Characteristics	1	2	3	4	5	6	7	8
Source of isolation	Faeces	Faeces	Spacecraft associated clean room	Marine coastal sediment	Grass soil[b]	Compost	Clean room air	Soil, Water
Cell shape	LR*	R	R	R	R	SR	R	R
Colony color	Cream	Cream	Cream	Cream	Light yellow[b]	Cream	Orange-Reddish	Cream
Cell size (μm)	5.8 × 1.3	4.0 × 1.4	3.9 × 1.6	1.5 × 0.6	1.2–2.5 × 0.7–0.8[b]	1.2 × 0.8	5.3 × 1.2	0.7–0.8 × 2.0–3.0
Endospore shape/location	E/T	E/T	E/T	E/T	E/C[b]	E/C-PC	S/C-ST	E-S/C-PC-ST
Oxidase	+	+	+	+	+	–	–	+
Motility	+	+	+	+	+	–	+	+
Temperature (°C)								
Range	15–50	25–50	25–50	25–55	10–60[b]	20–55	13–45[a]	5–55
Optimum	30	35–40	35–40	30–35	30[b]	50	30[a]	30
pH								
Range	5.0–9.0	5.0–9.0	5.0–9.0	5.0–9.0	5.0–10.0[b]	6.0–9.0	6.5–8.5[a]	5.5–8.5
Optimum	7.0	7.0–8.0	7.0–8.0	8.0	7.0[b]	6.5–7	7.0–8.0[a]	7.0–8.0
NaCl (%)								
Range	0–10.0	0–10.0	0–10.0	0–10.0	0–8.0	0–10.0	0–8.5[a]	2.0–10.0
Optimum	0–2.0	0.5–2.0	0.5–2.0	0–2.0	0–0.5	0.5–2.0	0.5[a]	0–2.0
Hydrolysis of:								
Starch	+	–	–	–	–	–	+	+
Gelatin	+	+	–	+	–	–	+	–
Casein	+	+	+	–	–	–	–	+
Esculin	–	–	–	–	–	–	+	+
API 20NE tests								
Nitrate reduction	–	–	–	+	+	+	–	+
Urease	–	–	–	+	+	–	–	–
β-galactosidase production	–	–	–	–	–	–	+	+
Assimilation of:								
N-Acetyl-glucosamine	+	+	–	+	–	+	–	+
Adipic acid	–	+	–	[+]	–	–	–	–
L-Arabinose	[+]	+	–	[+]	–	+	–	+
Capric acid	–	+	–	–	–	–	–	–
D-Glucose	[+]	+	–	[+]	–	+	–	+
Malic acid	+	+	+	+	–	+	–	+
D-Maltose	[+]	+	–	[+]	–	+	–	+
D-Mannitol	+	+	–	[+]	–	+	–	+
D-Mannose	+	–	–	[+]	–	+	–	+
Phenylacetic acid	+	+	+	+	+	–	–	–
Potassium gluconate	–	+	–	[+]	–	+	+	+
Trisodium citrate	[+]	–	–	–	+	–	–	+

Table 1 (*Continued*)

Characteristics	1	2	3	4	5	6	7	8
Acid production from:								
Arabinose	–	–	–	–	–	–	+	+
Cellobiose	–	–	–	–	–	–	+	+
Dextrose	–	–	–	–	–	+	+	–
Fructose	–	–	–	–	–	–	+	+
Galactose	–	–	–	–	–	–	+	–
Inulin	–	–	–	–	–	–	+	–
Lactose	–	–	–	–	–	–	+	–
Maltose	–	–	–	–	–	–	+	–
Mannitol	–	–	–	–	–	–	+	–
Mannose	–	–	–	–	–	–	+	+
Melibiose	–	–	–	–	–	–	+	–
Raffinose	+	–	–	–	–	–	+	–
Rhamnose	–	–	–	–	–	–	+	–
Salicin	–	–	–	+	–	–	+	–
Sorbitol	–	–	–	–	–	–	+	–
Sucrose	–	–	–	–	–	+	+	+
Trehalose	–	–	–	–	–	–	[+]	–
Xylose	–	–	–	–	–	–	+	–
API (ZYM)								
Acid phosphatase	–	–	[+]	[+]	–	–	–	+
Alkaline phosphatase	–	–	[+]	[+]	–	[+]	+	+
α-Chymotrypsin	–	–	[+]	[+]	–	–	+	[+]
Cystine arylamidase	–	–	–	–	–	–	+	–
Esterase	+	+	+	+	+	+	+	+
Esterase lipase	+	+	+	+	+	+	+	+
α-Galactosidase	–	–	–	–	–	–	–	+
β-Galactosidase	–	–	–	–	–	–	–	+
α-Glucosidase	–	–	–	–	–	–	–	+
β-Glucosidase	–	–	–	–	–	–	–	+
β-Glucuronidase	–	–	–	–	–	–	–	+
Leucine arylamidase	–	–	[+]	[+]	–	+	+	[+]
Naphthol-AS-BI-phosphohydrolase	–	+	+	[+]	+	+	[+]	+
Trypsin	–	–	–	–	–	–	+	[+]
Valine arylamidase	–	–	–	–	–	–	+	[+]
Major fatty acids (>10%)	iso-$C_{15:0}$, anteiso-$C_{15:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$	iso-$C_{15:0}$, iso-$C_{16:0}$, $C_{16:1}$ *ω7c* alcohol	iso-$C_{15:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$	iso-$C_{15:0}$, iso-$C_{17:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$
Menaquinone composition	MK-7	MK-7	MK-7	MK-7	MK-7[b]	MK-7, MK-6 (Tr)	MK-6[a]	MK-7
Major Polar lipids	DPG,PG, PE, APGL1	DPG, PG, PE, AL1, APGL1	DPG, PG, PE, APGL1	DPG, PG, PE, APGL1	DPG, PG, PE, APGL1	DPG, PG, PE, APL1, APGL1	DPG, PG, APGL2	DPG, PG, PE, GL1, PGL1
DNA G + C content (mol%)	43.9	43.6	43.8	44.0	41.2	44.3	42.7	43.5

All strains are positive for catalase. Negative for indole production, glucose fermentation, arginine dihydrolase, acid production from adonitol, dulcitol, inositol. Negative for production of enzymes N-acetyl-β-glucosaminidase, α-fucosidase, lipase and α-mannosidase.

*Please refer to Supplementary Fig. S7 for SEM image of the strain DSM 23^T.

[a] Data adopted from Seiler et al. [52].

[b] Data adopted from Liu et al. [31].

Table 2
Comparative fatty acid profiles of *Pseudobacillus badius* DSM 23^T (1), DSM 5610 (2), DSM 30822 (3), SGD-V-25 (4), *Pseudobacillus wudalianchiensis* DSM 100757^T (5), *Q. thermotolerans* SgZ-8^T (6), *Domibacillus robiginosus* DSM 25058^T (7) and *B. subtilis* subsp. *subtilis* DSM 10^T (8).

Fatty acid type	1	2	3	4	5	6	7	8
Saturated fatty acids								
$C_{14:0}$	2.5	1.9	2.3	2.3	2.5	2.2	1.2	Tr
$C_{16:0}$	6.7	5.1	5.4	5.1	4.8	5.6	7.8	4.1
Branched fatty acids								
iso-$C_{14:0}$	3.4	1.2	2	1.7	2.7	5.8	nd	1.6
iso-$C_{15:0}$	**42.1**	**46.0**	**48.6**	**47.1**	**39.4**	**15.5**	**11.6**	**20.7**
iso-$C_{16:0}$	8.4	4.7	5.8	5.3	5.8	**18.9**	Tr	5.8
iso-$C_{17:0}$	2.4	3	2.4	2.8	2.6	2.5	4.2	**10.9**
anteiso-$C_{15:0}$	**9.1**	**9.1**	**9.1**	**10.9**	**17.6**	8.1	**40.4**	**40.7**
anteiso-$C_{17:0}$	4.7	5.5	4.6	5.9	6.9	5.6	**29.9**	**13.3**
Unsaturated fatty acids								
iso-$C_{17:1}$ ω10*c*	3.6	6.7	4.4	4.4	2.7	4.3	nd	Tr
$C_{16:1}$ ω7*c* alcohol	4.2	3.1	3.9	3.1	3.2	**13.8**	nd	Tr
$C_{16:1}$ ω11*c*	3.1	5	3.4	3	3.0	9.1	3.1	Tr
Summed feature 3*	4.3	4	4.5	4.7	4.7	3.8	nd	nd
Summed feature 4*	2.5	4	2.7	3.2	2.0	2.4	1.1	nd

All data is from present study. The strains were cultivated on TSBA at 30° C for 24–48 h. Major fatty acids (≥10%) are indicated in bold font. nd-not detected. Tr-traces (<1.0%). Values are averages of three readings. *Summed feature 3 ($C_{16:1}\omega7c$/$C_{16:1}\omega6c$); Summed feature 4 ($C_{17:1}$ iso II/anteiso B).

annotation was carried out by RAST (Rapid Annotation using Subsystem Technology) [6,41], tRNA was predicted by tRNAscan-SE 1.23 [33] and rRNA genes by RNAmmer 1.2 [27]. The assembled genomes were annotated using the NCBI prokaryotic Genome Automatic Annotation Pipeline (PGAAP) (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/). The genome sequences of *B. badius* DSM 23^T and closely related *Domibacillus* spp. and *Quasibacillus* spp. were downloaded from the NCBI website (http://www.ncbi.nlm.nih-gov/genome). The genome-genome similarity between the strains was calculated using the parameters of original/ortho-ANI available in the software OAT [29] and digital DNA-DNA hybridization through the genome to genome distance calculator (GGDC) available at the link http://ggdc.dsmz.de/-distcalc2.php [35]. Based on the 16S rRNA gene sequence analysis, draft and finished genomes of the four strains of *B. badius* and close relatives were downloaded from NCBI. Putative taxonomy and microbial phylogeny of *B. badius* strains was also inferred using PhyloPhlAn version 0.99 [40] which utilizes database of >400 non redundant universal marker proteins which were optimized from more than 3700 available genomes from IMG v 3.4 [34]. The initial pre-processing steps in PhyloPhlAn include identification of core genes and merging these genes into universal protein families. This is followed by ranking of each protein family for covered diversity and ubiquitous conservation in microbial genomes, it also classifies genomes through closely related subspecies which enhances the consistency of taxonomic and phylogenetic grouping. A total of 38 genomes were downloaded from NCBI and converted to amino acid sequences using prodigal v 2.6.2 [21] which were then used as an user input in PhyloPhlAn to construct phylogenetic tree in which each sequence is aligned separately to >400 marker proteins using integrated MUSCLE algorithm v 3.8.1 [16]. Final reconstruction of tree was done using FastTree v 2.1.1 [44] and the tree was rendered in figtree v1.4.3 [45].

The minimum standards for description of endospore forming, Gram-staining-positive taxa were followed [32] with determination of phenotypic properties of *B. badius* DSM 23^T, DSM 30822, DSM 5610, SGD-V-25, *B. wudalianchiensis* DSM 100757^T and their closest phylogenetic relatives *D. robiginosus* DSM 25058^T, *Q. thermotolerans* SgZ-8^T and *B. subtilis* subsp. *subtilis* DSM 10^T (Table 1). The differences in phenotypic properties i.e. physiological parameters for growth and inability to ferment a majority of sugars like pentoses and hexoses compared to *D. robiginosus* DSM 25058^T were some major differences (Table 1). Further, within the four strains of *B. badius* and *B. wudalianchiensis* DSM 100757^T there were some variations with respect to colony color, cell size, polymer hydrolysis, nitrate reduction and pattern of oxidation of substrates in the GEN III omnilog system (however remarkably the four strains of *B. badius* and *B. wudalianchiensis* DSM 100757^T revealed an unifying phenotypic fingerprint in 94 tests of the omnilog system, Fig. S9). Some of these differences might be attributed to the different habitats of isolation of the strains and thus their adaptation, representing intra- and inter-species diversity (Table 1 and Supplementary Table S3).

The riboprint pattern clustered the strains *B. badius* DSM 23^T, DSM 5610, 30822, SGD-V-25 and *B. wudalianchiensis* DSM 100757^T into a separate group distinct from *Bacillus sensu stricto*, genera *Domibacillus* and *Quasibacillus* (Supplementary Fig. S5). The patterns revealed that *B. wudalianchiensis* DSM 100757^T differed slightly from the other four *B. badius* strains in its profile and all the five strains within this cluster had some unique differentiating bands (Supplementary Fig. S5). The MSP dendrogram of MALDI-TOF peaks complemented the findings of riboprinting wherein the four strains of *B. badius* and *B. wudalianchiensis* DSM 100757^T fell nicely into a separate group distinct from other *Bacillus* spp., *Domibacillus* spp., and *Quasibacillus* spp. (Supplementary Fig. S6). Similar to riboprinting, the protein profiling of *B. wudalianchiensis* DSM 100757^T differed from the other four strains of *B. badius*. Thus both riboprinting and MALDI-TOF MS complemented the phenotypic analyses and supported its findings of inter- and intra-species diversity within *B. badius* and between the latter and *B. wudalianchiensis* DSM 100757^T.

Chemotaxonomic data such as fatty acids revealed a distinct pattern in the strains *B. badius* DSM 23^T, DSM 5610, DSM 30822, SGD-V-25 and *B. wudalianchiensis* DSM 100757^T with iso-$C_{15:0}$ (39.4–48.6%) as the predominant fatty acid and moderate amounts of anteiso-$C_{15:0}$ (9.1–17.6%) (Table 2). In comparison *Q. thermotolerans* SgZ-8^T, *D. robiginosus* DSM 25058^T and *B. subtilis* subsp. *subtilis* DSM 10^T showed substantially lower amounts of iso-$C_{15:0}$ (11.6–20.7%) and the latter two strains showed higher amounts of anteiso-$C_{15:0}$ (40.4–40.7%) and anteiso-$C_{17:0}$ (13.3–29.9%) (Table 2). Further *Q. thermotolerans* SgZ-8^T contained iso-$C_{16:0}$ (18.9%) and $C_{16:1}\omega7c$ alcohol (13.8%) as the predominant fatty acid and moderate amounts of $C_{16:1}\omega11c$ which were in relatively lower amounts in *B. badius* DSM 23^T, DSM 5610, DSM 30822 and *B. wudalianchiensis* DSM 100757^T. These variations in the fatty acid profiles are collated in the hierarchical cluster analyses of the strains (Supplementary Fig. S8). Analyses of two-dimensional polar lipids profile of strains *B. badius* DSM 23^T, 5610, 30822, SGD-V-25 and *B. wudalianchiensis* DSM 100757^T with their closest taxa revealed presence of diphosphatidylglycerol (DPG), phosphatidylglycerol

Table 3
Comparison of differential characteristics of the genus *Pseudobacillus* with the related taxa and the type species of *Quasibacillus*, *Domibacillus* and *Bacillus*.

Characteristics	*Pseudobacillus*	*Quasibacillus*[a]	*Domibacillus*[b,c]	*Bacillus*[d]
Endospore formation	E	O-E	S, S-E, ST-T	E, (S)
Oxidase	+	–	Variable	Variable
Oxidation of α-D-glucose	–	–	+	+
Temperature growth range (°C)	10–60	15–55	10–45	30–65
Menaquinone system	MK-7	MK-7	MK-6, 7	MK-7
Major fatty acids	iso-$C_{15:0}$ and anteiso-$C_{15:0}$	iso-$C_{15:0}$, iso-$C_{16:0}$, $C_{16:1}\omega7c$ alcohol	iso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{17:0}$
Polar lipids	DPG,PG, PE, APGL	DPG, PG, PE	DPG, PG	DPG, PG, PE, GBG, APL
Cell wall type	meso-Dpm direct	meso-Dpm direct	meso-Dpm direct	meso-Dpm direct (D)
DNA G + C content (mol%)	41.2-44	44.2-44.3	40.6-46.5	32.0-69.0
16S rRNA gene sequence similarity with *B. subtilis* subsp. *subtilis* DSM 10^T (%)	93	94	93	100

Data from present analysis, as otherwise mentioned. +, positive; –, negative; E, ellipsoidal; S, spherical; O, oval; ST, sub-terminal; T, terminal; DPG, diphosphatidyl- glycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; APGL, aminophosphoglycolipid; APL, aminophospholipid; GBG, gentiobiosyldiacylglycerol; D, different; in parentheses, rare or not specified in the description of the genus.
[a] Verma et al. [58].
[b] Verma et al. [59].
[c] Verma et al. [60].
[d] Kämpfer et al. [23], except the ones in italic font which are described from this study.

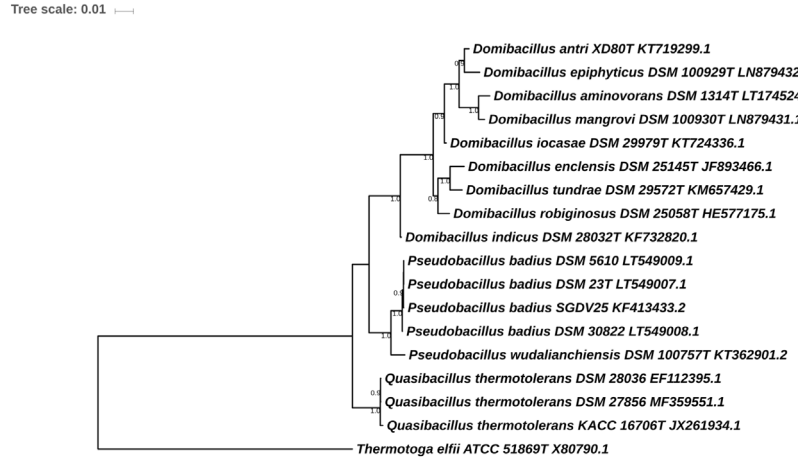

Fig. 1. Evolutionary relationship of *Pseudobacillus* strains with members of *Bacillaceae* family based on 16S rRNA gene.
The evolutionary history was inferred using the approximately-maximum-likelihood FastTree version 2.1 [44]. The optimal tree with the sum of branch length = 2.569 is shown with GTR rates (ac ag at cg ct gt) 0.8673 2.2434 0.9476 0.7096 3.9406 1.0000. Nucleotide evolution model used is Jukes-Cantor with balanced Support 1000 [22]. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. The final image was rendered in iTOL [30]. The phylogeny of *Pseudobacillus* strains with all the members of *Bacillaceae* family has been shown in Supplementary Fig. S2.

(PG), phosphatidylethanolamine (PE) and an aminophosphoglycolipid (APGL1) as the major lipids with minor quantities of a phospholipid (PL2) and an unknown lipid (Supplementary Fig. S1 and Table S1). The closest genera *Quasibacillus* and *Domibacillus* did not contain APGL1 but showed the presence of APGL2 (Supplementary Fig. S1; Table S1). Further the genus *Quasibacillus* contained the major lipid phosphatidylmonomethylethanolamine (PME) and the minor lipids APL1 and few unknown lipids (UL2-5) that were absent in *B. badius*, *B. wudalianchiensis* DSM 100757^T and *Domibacillus* (Supplementary Fig. S1; Table S1). The strain *B. subtilis* subsp. *subtilis* DSM 10^T exclusively showed the presence of the glycolipids (GL2, 3) and a phosphoglycolipid (PGL1) that could not be detected in any other strain. The profile was found to be more and less in aggrement with the previous reports [23, 37]. Further there were variations in the minor lipid profiles (PL1 and AL1) within the four strains of *B. badius* that reflect the intraspecies diversity. *B. wudalianchiensis* DSM 100757^T showed the presence of unique lipids (APL2 and PL4) which could not be detected in any of the *B. badius* strains. The profiles of *B. wudalianchiensis* DSM 100757^T in our analysis was found to have some differences from earlier report [31] in terms of minor lipid components with the spots originally labeled as aminophospholipid (APL) and phospholipid (PL2) detected as aminophosphophoglycolipid (APGL1) and aminophospholipid (APL2) in our analysis respectively (Supplementary Fig. S1; Table S1). This could be due to application of different methods of extraction and detecting lipids in the two studies. Whole cell peptidoglycan analyses of the four strains of *B. badius* and *B. wudalianchiensis* DSM 100757^T revealed meso-Dpm as the diagnostic diamino acid suggesting an A1γ type peptidoglycan (type A32.1) according to www.peptidoglycan-types.info (Table 4). MK-7 was the only quinone that could be detected in the four *B. badius* strains and *B. wudalianchiensis* DSM 100757^T (Table 1). On a whole, the chemotaxonomic analyses revealed distinct fatty acid and polar lipids profiles of the four *B. badius* strains and *B. wudalianchiensis* DSM 100757^T which demarcates them from the genera *Quasibacillus*, *Domibacillus* and *Bacillus* (Table 2; Supplementary Figs S1, S8; Table S1).

The four strains *B. badius* DSM 23^T, DSM 5610, DSM 30822 and SGD-V-25 showed high pairwise 16S rRNA gene sequence similarity values of 99.9-100% between themselves. The type strain *B. badius* DSM 23^T showed highest 16S rRNA gene sequence similarity with recently described strain *B. wudalianchiensis* DSM 100757^T (98.7%) followed by *Q. thermotolerans* SgZ-8^T (96.5%) and *D. robiginosus* DSM 25058^T (95.9%). Similarity to *B. subtilis* subsp.

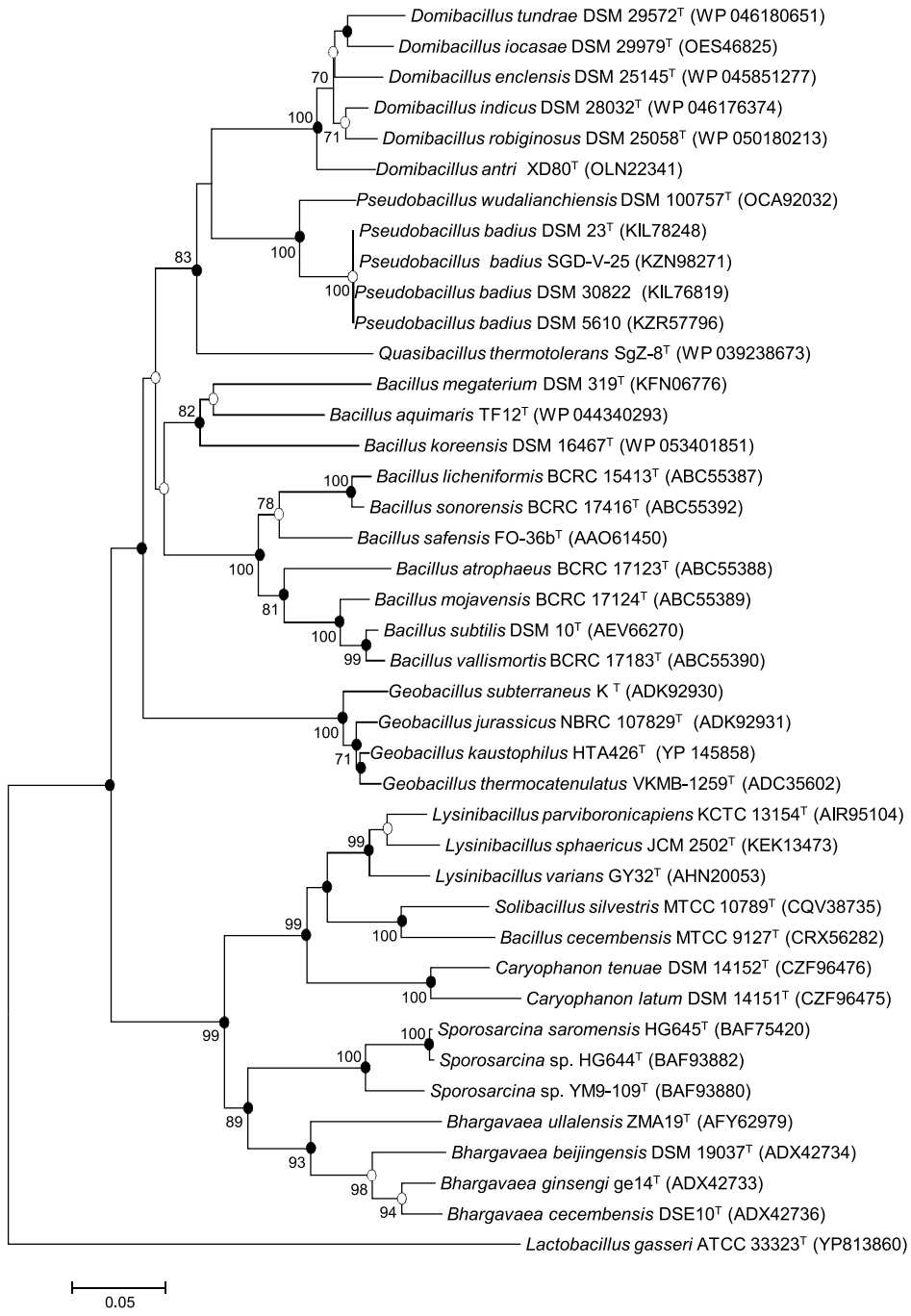

Fig. 2. Evolutionary relationships of taxa based on GyrB amino acid sequence.
The evolutionary history was inferred using the Neighbor-Joining method [48]. The optimal tree with the sum of branch length = 2.14529682 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches [19]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method [39] and are in the units of the number of amino acid differences per site. The analysis involved 40 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 313 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [55]. (●) symbols represent nodes that were also recovered in maximum-likelihood and parsimony approaches whereas (○) symbol represents those also recovered in maximum-likelihood only. Bar represents 0.05 substitutions per site.

subtilis DSM 10^T (type species of the genus *Bacillus*) was very low (93.3%). Phylogenetic analysis based on approximately maximum-likelihood analysis of 16S rRNA gene sequences retrieved from all the representative genera of the family *Bacillaceae* revealed that the four *B. badius* strains and *B. wudalianchiensis* DSM 100757^T branched in a separate clade closest to the genera *Domibacillus* and *Quasibacillus*, with *Bhargavaea cecembensis*, *Falsibacillus pallidus* and *Bacillus megaterium* forming part of a bigger group (Supplementary Fig. S2) when the evolutionary phylogeny was inferred through FastTree version 2.1.1 [44]. The species group *Bacillus sensu stricto* (as per recommendations of Bhandari et al. [9]), was well separated from this clade and members of the family *Planococcaceae* and *Bacillus* rRNA group 2 consisting of the genera *Filibacter*, *Sporosarcina*, *Paenisporosarcina*, *Psychrobacillus*, *Chryseomicrobium*, *Lysinibacillus*, *Kurthia*, *Caryophanon*, *Solibacillus* etc. (Supplementary Fig. S2). Interestingly, trees constructed using NJ, MP and

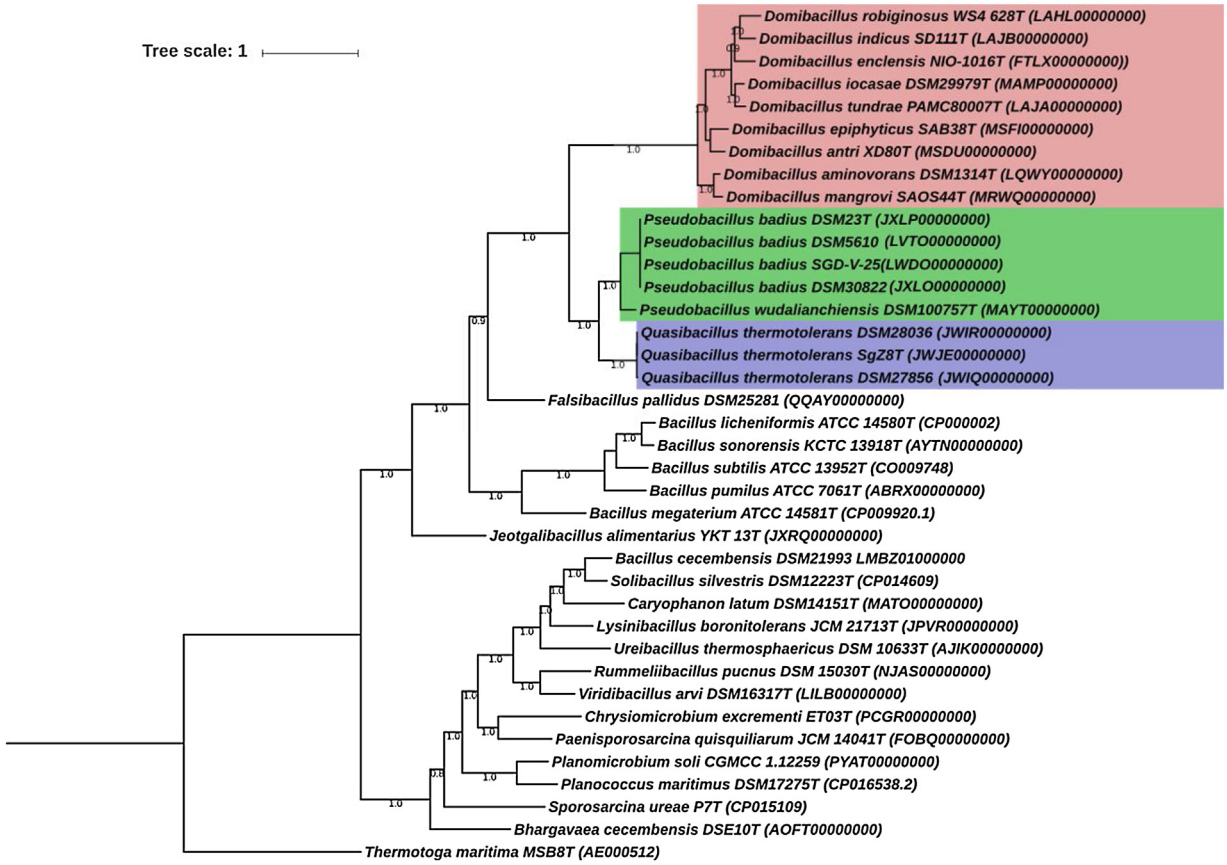

Fig. 3. Evolutionary relationship among the *Pseudobacillus* strains and related reference taxa using PhyloPhlAn version 0.99.
Phylogenetic tree showing the predicted phylogenetic relationship of representatives of *Pseudobacillus badius* and phylogenetically related taxa based on approximately 400 conserved bacterial marker proteins. The details of the methods are given in the text. Final tree construction was done using FastTree v2.1.1 [44] with JTT substitution model using local support values according to the Shimodaira-Hasegawa test. Bar represents tree scale 0.5.

ML methods incorporated within MEGA6.0 software retrieved the same branching pattern (data not shown). In order to better show the phylogenetic distance among the *Pseudobacillus* strains, only the members of *Domibacillus* and *Quasibacillus* were taken into consideration along with the outgroup (Fig. 1). A look at the GyrB amino acid sequence analysis further clarified the taxonomic status of the four *B. badius* strains and *B. wudalianchiensis* DSM 100757^T since being a faster evolving housekeeping gene than the SSU rRNA it provides a better resolution. At the GyrB amino acid sequence level the four strains showed 100% sequence match thus reinforcing the affiliation of the strains to the same species with low sequence similarity to *B. wudalianchiensis* DSM 100757^T (95%), followed by *Q. thermotolerans* SgZ-8^T (86.4%) and members of the genus *Domibacillus* (<82.5%). Similarity to *B. subtilis* subsp. *subtilis* DSM 10^T was extremely low (<80.0%) indicating that these strains do not belong to the genus *Bacillus*. Similar levels of GyrB amino acid sequence identities have been obtained in earlier studies of reclassification of *Bacillus* spp. into other genera [56]. Phylogenetic analysis separated the four *B. badius* strains and *B. wudalianchiensis* DSM 100757^T into a discrete group from genus *Domibacillus* whereas *Q. thermotolerans* was retrieved as a further distant neighbour of the four strains and the topology of the tree is more or less in agreement in all the tree making algorithms and characteristically similar to 16S rRNA gene based phylogeny (Fig. 2). The phylogenetic analysis based on *gyrB* nucleotide sequences also retrieved the same branching pattern except that the genus *Quasibacillus* was retrieved along with members of *B. badius* strains, while the members of the genus*Domibacillus* formed a different cluster similar in line with the amino acid sequences (Supplementary Fig. S3). This indicated that the four *B. badius* strains belong to the same species and *B. wudalianchiensis* DSM 100757^T is a member of a separate species. Further data from molecular phylogenetic analyses of 400 conserved genes comfortably placed the four *B. badius* strains and *B. wudalianchiensis* DSM 100757^T into a genus level cluster separated from the genera *Domibacillus* and *Quasibacillus* (Fig. 3). This evolutionary pattern bears a striking resemblance to both 16S rRNA gene based and GyrB amino acid sequence based trees with *B. badius*, genus *Domibacillus* and *Q. thermotolerans* distantly related to *Bacillus sensu stricto* (Figs 1 and 2). All these phylogenomic analyses reinforce the view that the four strains of *B. badius* and the recently described *B. wudalianchiensis* DSM 100757^T belong to a new genus.

Analysis of whole genome sequences of strains *B. badius* DSM 23^T, DSM 5610, DSM 30822 and SGD-V-25 revealed that they share >70% (81.6–96.8%) and >95% (97.8–99.6%), GGDC and ANI (both original and ortho) values respectively between themselves (Supplementary Table S2), thus representing strains of the same species [20,29,35,46,54,61]. It is also pertinent to note that the mol% G+C content difference between the four *B. badius* strains is only 0.4% (43.6–44.0%) which is well below the threshold of 1% maximum variation found for strains belonging to the same species for which the genomic DNA G+C contents have been determined from whole genome sequences [36]. Further comparison of the genome sequences of the four strains showed that they shared low GGDC and ANI values with *B. wudalianchiensis* DSM 100757^T (GGDC: 22.3–22.4 & ANI: 78.4–78.6) and members of the genera *Domibacillus* (GGDC: 20.3–22.3 & ANI: 68.9–69.8) and *Q. thermotolerans* SgZ-8^T (GGDC: 19.3–20.3 & ANI: 72.8–73.3). These values are well below the proposed cut-off for delineating bacte-

Table 4
Description of *Pseudobacillus* gen. nov. (GA00062), *Pseudobacillus badius* comb. nov. (TA00142) and *Pseudobacillus wudalianchiensis* comb. nov. (TA00800) according to digital protologue assigned by the www.imedea.uib.es/dprotologue website. The values shown in brackets [] in the last column represents the tests being done in the present study.

Taxonumber (TXNR)	GA00062	TA00142	TA00800
Former Taxonumber of the protologues subjected to emendation (FTXN)			TA00501
Species name (SPNA)		*Pseudobacillus badius*	*Pseudobacillus wudalianchiensis*
Genus status (GENA)	comb. nov.		
Genus name (GENA)	*Pseudobacillus*		
Genus etymology (GETY)	Pseu.do.ba.cil'lus. Gr. adj. pseudes false; L. masc. n. *Pseudobacillus* a false bacillus, because it is closely related to this genus and wrongly identified as Bacillus		
Type species of the genus (GENT)	*Pseudobacillus badius*		
Taxonumber of the type species (TXNS)	TA00142		
Specific epithet (SPEP)		*badius*	*wudalianchiensis*
Species status (SPST)		comb. nov.	comb. nov.
Species etymology (SPTY)		ba'di.us. L. masc. adj chestnut brown	wu.da.li.an.chi.en'sis. N.L. masc. adj. *wudalianchiensis* pertaining to Wudalianchi in Heilongjiang Province
Authors (AUTH)	Verma A, Pal Y, Ojha, AK, Kumari, M, Khatri I, Natarajan RK, Schumann P, Dastager SG, Mayilraj S, Subramaniam S, Krishnamurthi S		Verma A, Pal Y, Ojha, AK, Kumari, M, Khatri I, Natarajan RK, Schumann P, Dastager SG, Mayilraj S, Subramaniam S, Krishnamurthi S
Title (TITL)	Taxonomic insights into the phylogeny of *Bacillus badius* and proposal for its reclassification to the genus *Pseudobacillus* as *Pseudobacillus badius* comb. nov. and reclassification of *Bacillus wudalianchiensis* Liu et al., 2017 as *Pseudobacillus wudalianchiensis* comb. nov.		Taxonomic insights into the phylogeny of *Bacillus badius* and proposal for its reclassification to the genus *Pseudobacillus* as *Pseudobacillus badius* comb. nov. and reclassification of *Bacillus wudalianchiensis* Liu et al., 2017 as *Pseudobacillus wudalianchiensis* comb. nov.
Submitter (SUBM)	Ashish Verma	Srinivasan Krishnmaurthi	Ashish Verma
e-mail of the submitter (EMSU)	ashish.csirihbt@gmail.com	kmurthi@imtech.res.in/ kinutaxon@gmail.com	ashish.csirihbt@gmail.com
Designation of the type strain (TYPE)	DSM 23	DSM 23	FJAT-27215
Strain collection numbers (COLN)	DSM 23 = ATCC 14574 = CCM 2113 = NCIB 9364 = NCTC 10333		DSM 100757 = CCTCC AB 2015266
16S rRNA gene accession number (16SR)	X77790	X77790	KT362901
Alternative housekeeping genes:gene [accession number] (HKGN)	*gyrB* [KIL78248]	gyrB [KIL78248]	gyrb [OCA92032]
Genome accession number [RefSeq]	NZ_JXLO00000000.1	NZ_JXLO00000000.1	NZ_MAYT00000000.1
Genome status (GSTA)	Draft	Draft	Draft
Genome size (GSIZ)	4051	4051	4427
GC mol % (GGCM)	43.9	43.9	41.2
Country of origin (COUN)	USA	USA	China
Region of origin (REGI)	Baltimore	Baltimore	Wudalianchi in the Heilongjiang Province
Date of isolation (DATI)	1919/01/01		
Source of isolation (SOUR)	Faeces	Faeces	Grass soil
Sampling date (DATS)	1919/01/01	1919/01/01	2016-05-02
Geographic location (GEOL)			Wudalianchi scenic area, China
Latitude (LATI)			43°26′N–53°33′N
Longitude (LONG)			121°11′E–135°5′E
Number of strains in study (NSTR)	5 (DSM 23^T, DSM 5610, DSM 30822, SGD-V-25 and DSM 100757^T)	4 (DSM 23^T, DSM 5610, DSM 30822 and SGD-V-25)	1 (DSM 100757^T)
Source of isolation of non-type strains (SAMP)	Faeces, spacecraft associated clean room and marine coastal sediment	Faeces, spacecraft associated clean room and marine coastal sediment	
Growth medium, incubation conditions [Temperature, pH, and further information] used for standard cultivation (CULT)	Tryptic Soya broth agar [temp 30° C, pH 7.0, 48 h incubation]	Tryptic Soya broth agar [temp 30° C, pH 7.0, 48 h incubation]	Luria-Bertani Agar, 30° C, optimum pH 7.0
Conditions of preservation (PRES)	Lyophilization and −70° C storage in 10% glycerol	Lyophilization and −70° C storage in 10% glycerol	Lyophilization and −70° C storage in 10% glycerol
Gram stain (GRAM)	Positive	Positive	Positive
Cell shape (CSHA)	Rod	Rod	Rod
Cell size (length or diameter) (CSIZ)	1.2–5.8 × 0.7–1.3 μm	5.8 × 1.3 μm	1.2–2.5 × 0.7–0.8 μm
Motility (MOTI)	Motile	Motile	Motile
If motile (MOTK)			Flagellar
If flagellated (TFLA)			Lateral
Sporulation (resting cells) (SPOR)	Endospores	Endospores	Endospores
Location of spores (SPOL)	Terminal	Terminal	Central
Sporangium swollen (SPOW)		No	Yes

Table 4 (*Continued*)

Taxonumber (TXNR)	GA00062	TA00142	TA00800
Colony morphology (COLM)	Cells form cream coloured colonies with pinpoint to 0.2 mm diameter on tryptic soya broth agar after 24–48 h at 30° C	Cells form cream coloured colonies with pinpoint to 0.2 mm diameter on tryptic soya broth agar after 24–48 h at 30° C	Cells form circular and smooth light yellow colonies with 2–3 mm diameter on LB medium after 24 h at 30° C
Temperature range (TEMR)	10–60	15–50	10–60
Lowest temperature for growth (TEML)	10	15	10
Highest temperature for growth (TEMH)	60	50	60
Temperature optimum (TEMO)	30	30	30
Lowest pH for growth (PHLO)	5.0	5.0	5.0
Highest pH for growth (PHHI)	10.0	9.0	10.0
pH optimum (PHOP)	7.0	7.0	7.0
Lowest NaCl concentration for growth (SALL)	0	0	0
Highest NaCl concentration for Growth (SALH)	10.0	10.0	3.0 [8.0]
Salinity optimum (SALO)	0–2.0	0–2.0	0 [0–0.5]
Relationship to O_2 (OREL)	Aerobe	Aerobe	Aerobe
Positive tests with BIOLOG (BIOP)	**Positive:** L-alanine, β-hydroxy-D, L-butyric acid, sodium butyrate, α-keto-glutaric acid, guanidine HCl, lithium chloride, sodium lactate (1%), 1-8% NaCl, nalidixic acid, glycyl-L-proline, pH 5.0, pH 6.0, potassium tellurite, sodium butyrate **Weakly positive:** acetoacetic acid, L-aspartic acid, sodium bromate, dextrin, gelatin, L-glutamic acid, p-hydroxy-phenylacetic acid, inosine, tetrazolium blue, tetrazolium violet	**Positive:** L-alanine, β-hydroxy-D, L-butyric acid, sodium butyrate, α-keto-glutaric acid, glycerol, guanidine HCl, L-lactic acid, lithium chloride, sodium lactate (1%), 1-8% NaCl, nalidixic acid, glycyl-L-proline, pH 5.0, pH 6.0, potassium tellurite, sodium butyrate, pH 5.0, **Weakly positive:** acetoacetic acid, acetic acid, L-aspartic acid, L-arginine, aztreonam, sodium bromate, α-hydroxy-butyric acid, γ-amino-butyric acid, dextrin, formic acid, guanidine HCl, gelatin, glucuronamide, L-glutamic acid, inosine, nalidixic acid, N-acetyl neuramininc acid, L-pyroglutamic acid, p-hydroxy-phenylacetic acid, bromo-succinic acid, L-serine, stachyose, D-serine, tetrazolium blue, tetrazolium violet	**[Positive:** L-alanine, L-aspartic acid, D-cellobiose, citric acid, dextrin, D-fructose, gentiobiose, D-gluconic acid, α-D-glucose, L-glutamic acid, glycerol, β-methyl-D-glucoside, myo-inositol, guanidine HCl, L-lactic acid, lithium chloride, sodium lactate (1%), 1-8% NaCl, D-maltose, D-mannitol, L-malic Acid, D-mannose, mucic acid, nalidixic acid, potassium tellurite, D-saccharic Acid, D-salicin, D-sorbitol, sodium butyrate, sucrose, D-trehalose **Weakly positive:** acetoacetic acid, L-aspartic acid, D-aspartic acid, L-arginine, γ-amino-butyric acid, D-fructose-6-phosphate, formic acid, D-galacturonic acid, gelatin, L-glutamic acid, N-acetyl-β-D-glucosamine, glucuronamide, D-glucuronic acid, L-histidine, inosine, D-melibiose, D-raffinose, L-rhamnose, stachyose, bromo-succinic acid, sodium bromate, tetrazolium blue, tetrazolium violet]
Negative tests with BIOLOG (BION)	D-arabitol, D-aspartic acid, α-keto-butyric acid, D-cellobiose, D-fucose, fusidic acid, gentiobiose, D-gluconic acid, α-D-glucose, 3-methyl glucose, D-glucose-6-phosphate, N-acetyl-β-D-galactosamine, N-acetyl-β-D-glucosamine, β-methyl-D-glucoside, myo-inositol, α-D-lactose, D-lactic acid methyl ester, D-malic acid, N-acetyl-β-D-mannosamine, L-malic acid, D-maltose, D-mannitol, D-mannose, D-melibiose, minocycline, mucic acid, niaproof 4, pectin, methyl pyruvate, propionic acid, L-rhamnose, rifamycin SV, D-saccharic acid, D-salicin, D-sorbitol, sucrose, D-serine, D-trehalose, D-turanose, troleandomycin, vancomycin	D-arabitol, α-keto-butyric acid, D-cellobiose, D-fructose, D-fucose, fusidic acid, gentiobiose, α-D-glucose, 3-methyl glucose, N-acetyl-β-D-galactosamine, N-acetyl-β-D-glucosamine, β-methyl-D-glucoside, D-galactose, myo-inositol, D-lactose, lincomycin, N-acetyl-β-D-mannosamine, D-maltose, D-mannitol, D-mannose, D-melibiose, minocycline, mucic acid, niaproof 4, methyl pyruvate, D-raffinose, rifamycin SV, D-salicin, D-serine, D-sorbitol, sucrose, D-trehalose, D-turanose, troleandomycin, vancomycin	[D-arabitol, D-aspartic acid, α-keto-butyric acid, D-cellobiose, D-fucose, fusidic acid, gentiobiose, α-D-glucose, 3-methyl glucose, D-glucose-6-phosphate, N-acetyl-β-D-galactosamine, N-acetyl-β-D-glucosamine, β-methyl-D-glucoside, D-galactose, glycerol, L-lactic acid, D-lactic acid methyl ester, D-lactose, D-malic acid, L-malic acid, D-maltose, D-mannitol, D-mannose, D-melibiose, minocycline, mucic acid, niaproof 4, methyl pyruvate, N-acetyl-β-D-mannosamine, N-acetyl neuramininc acid, pectin, propionic acid, L-pyroglutamic acid, pH 6.0, quinic acid, L-rhamnose, rifamycin SV, bromo-succinic acid, D-saccharic acid, D-salicin, D-serine, D-sorbitol, stachyose, sucrose, D-trehalose, D-turanose, troleandomycin, vancomycin]
Positive tests with API (APIP)	**API ZYM**: esterase, esterase lipase **API 20NE**: phenylacetic acid	**API ZYM**: esterase, esterase lipase **API 20NE**: N-acetyl glucosamine, malate, D-mannose, D-mannitol, phenylacetic acid	**API 20E:** arginine dihydrolase, citrate utilization, nitrate reduction, urease **[API ZYM**: esterase, esterase lipase, naphthol-AS-BI-phosphohydrolase **API 20NE**: Nitrate reduction, Urease, phenylacetic acid, trisodium citrate]

Table 4 (*Continued*)

Taxonumber (TXNR)	GA00062	TA00142	TA00800
Negative tests with API (APIN)	**API ZYM:** cystine arylamidase, leucine arylamidase, valine arylamidase, α-chymotrypsin, α-galactosidase, α-glucosidase, β-galactosidase, β-glucosidase, β-glucuronidase, acid phosphatase, alkaline phosphatase, trypsin **API 20NE:** adipic acid, capric acid, β-galactosidase production, potassium gluconate	**API ZYM**: N-acetyl-β-glucosaminidase, cystine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase,lipase, α-mannosidase, trypsin and valine arylamidase **API 20NE**: Reduction of nitrate, urease, β-galactosidase, adipate, caprate, gluconate **API 20E**: arginine, ornithine, lysine, citrate, H_2S production	**API 20E:** β-galactosidase, gelatinase, H2S production, indole, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, Voges-Proskauer **API 50CH:** arbutin, amygdalin, D-arabinose, L-arabinose, D-adonitol, N-acetylglucosamine, amidon, D-cellobiose, dulcitol, erythritol, esculin ferric citrate, D-fructose, D-fucose, glycogen, D-galactose, gentiobiose, D-glucose, glycerol, methyl-α-D-glucopyranoside, inulin, inositol, D-lactose, D-lyxose, D-maltose, D-mannose, D-mannitol, methyl-α-D-mannopyranoside, D-melibiose, D-melezitose, L-rhammose, D-raffinose, D-ribose, L-sorbose, D-saccharose, salicin, D-sorbitol, D-trehalose, D-turanose, D-tagatose, D-xylose, L-xylose, methyl-β-D-xylopyranoside, xylitol **[API ZYM**: acid phosphatase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, leucine arylamidase, trypsin valine arylamidase **API 20NE**: β-galactosidase, N-acetylglucosamine, adipic acid, L-arabinose, capric acid, D-glucose, malic acid, D-maltose, D-mannitol, D-mannose, potassium gluconate]
Acid formation for carbohydrates (all negative) (AFCN)	arabinose, cellobiose, dextrose, fructose, galactose, inulin, lactose, maltose, mannitol, mannose, melibiose, rhamnose, salicin, sorbitol, sucrose, trehalose, xylose	arabinose, cellobiose, dextrose, fructose, galactose, inulin, lactose, maltose, mannitol, mannose, melibiose, raffinnose, rhamnose, salicin, sorbitol, sucrose, trehalose, xylose	[arabinose, cellobiose, dextrose, fructose, galactose, inulin, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, xylose]
Commercial kits used? (KITA)	API 20NE, API ZYM, GEN III Microplate	API ZYM, 20NE, 20E, GEN III Microplate	API 20E, API 50CH [API 20NE, API ZYM, GEN III Microplate]
Oxidase (OXID)	Positive	Positive	Negative [positive]
Catalase (CATA)	Positive	Positive	Positive
Quinone type (QUIN)	MK-7	MK-7	MK-7
Major fatty acids (FAME)	iso-$C_{15:0}$ and anteiso-$C_{15:0}$	iso-$C_{15:0}$ and anteiso-$C_{15:0}$	iso-$C_{15:0}$ and anteiso-$C_{15:0}$
Peptidoglycan type (PGLC)	A1γ with meso-Dpm as the diagnostic diamino acid	A1γ with meso-Dpm as the diagnostic diamino acid	A1γ with meso-Dpm as the diagnostic diamino acid
Phospolipid pattern or diagnostic phospolipid (PHOS)	Diphosphatidylglycerol (DPG), Phosphatidylglycerol (PG), Phosphatidylethanolamine (PE), Aminophosphoglycolipid (APGL)		Diphosphatidylglycerol (DPG), Phosphatidylglycerol (PG), Phosphatidylethanolamine (PE) [Diphosphatidylglycerol (DPG), Phosphatidylglycerol (PG), Phosphatidylethanolamine (PE), Aminophosphatidylglycerol (APGL), Aminophospholipids (APL)]
Biosafety level (BIOS)	00001	00001	00001
Habitat (HABT)			ENVO:00005750

rial species [20,29] and indicate that the strains *B. badius* DSM 23^T, DSM 5610, DSM 30822 and SGD-V-25 are members of the same species and *B. wudalianchiensis* DSM 100757^T is a separate species. The dendrogram based on ANI values clustered *B. badius* and *B. wudalianchiensis* DSM 100757^T into a single clade distinct from the genera *Quasibacillus* and *Domibacillus* (Supplementary Fig. S4).

Our research in this manuscript hypothesized that the four strains of *B. badius* DSM 23^T, DSM 5610, DSM 30822 and SGD-V-25 and the recently proposed *B. wudalianchiensis* DSM 100757^T should be reclassified as members of a novel genus *Pseudobacillus* as two separate species. Data from phenotypic, MALDI-TOF and riboprint, support our conclusions and indicate good intra and inter-species diversity (Table 1, Supplementary Figs S5 and S6). Most importantly, phylogenetic analyses from sequences of 16S rRNA and *gyrB* gene, GyrB amino acid and 400 conserved genes arrive at the same conclusion and strengthen the notion of a novel genus status for the four *B. badius* strains and *B. wudalianchiensis* DSM 100757^T (Figs 1, 2, 3; Supplementary Figs S2 and S3). This evolutionary pattern is also strongly supported by hierarchal clustering observed using C-source oxidation pattern (Omnilog) and fatty acid data (Supplementary Figs S9) which delineate *B. badius* strains and *B. wudalianchiensis* DSM 100757^T into a single clade. Finally chemotaxonomic analyses (especially fatty acids and polar lipids) have revealed specific markers for the genus *Pseudobacillus* (Table 2 and Supplementary Table S1, Figs S1 and S8). The differentiating characteristics between the genera *Pseudobacillus, and Quasibacillus*, *Domibacillus* and *Bacillus* are summarized in Table 3. Thus the four strains *B. badius* DSM 23^T, DSM 5610, DSM 30822, and SGD-V-25 are classified as members of a single species of a novel genus *Pseudobacillus* as *P. badius* comb. nov. and *B. wudalianchiensis*

DSM 100757^T is reclassified as *P. wudalianchiensis* comb. nov. The digital protologue for *Pseudobacillus* gen. nov., *P. badius* comb. nov. and *P. wudalianchiensis* comb. nov. has been mentioned in Table 4.

New taxa-*Firmicutes*

The NCBI accession numbers for the draft genome sequences of strain *B. badius* DSM 23^T, DSM 5610, DSM 30822 and SGD-V-25 are JXLP00000000, LVTO00000000, JXLO00000000 and MAIG00000000 respectively.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2019.03.003.

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