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Reclassification of [*Haemophilus*] *haemoglobinophilus* as *Canicola haemoglobinophilus* gen. nov., comb. nov. including Bisgaard taxon 35

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Running title: *Canicola haemoglobinophilus* gen. nov.

16S rRNA gene sequences determined in the present investigation have been deposited with GenBank/EMBL/DDBJ under the accession numbers MW396726-MW396730 for 5 strains. Partial *rpoB* gene sequences for 14 strains have been deposited under the numbers MW400941-MW400954. The *recN* sequences for 4 strains have been deposited under the numbers MW400936-MW400939. The whole genome sequence of strain CCUG 16472 has been deposited with the accession number JAEOAI01 (supplementary Table 1).

Abstract

[*Haemophilus*] *haemoglobinophilus* and the unpublished Bisgaard taxon 35 are associated with respiratory and urogenital tract infections in dogs. Twenty-one strains including the type strain of [*Haemophilus*] *haemoglobinophilus* were included in the investigation. Strains of [*Haemophilus*] *haemoglobinophilus* and taxon 35 formed a monophyletic group demonstrating at least 97.8 and 96.5% similarities within the group

based upon 16S rRNA and *rpoB* gene sequence comparisons, respectively. *Glaesserella australis* was the closest related species to [*Haemophilus*] *haemoglobinophilus* and taxon 35 with 96.1% 16S rRNA gene sequence similarity which is slightly higher than the 95% separating most genera of *Pasteurellaceae*. However, the conserved protein sequence phylogeny documented a unique position of [*Haemophilus*] *haemoglobinophilus* with only 81% identity to the closest related species, genomospecies 1 of *Rodentibacter* which is lower than the 85% separating most genera of *Pasteurellaceae.* The conserved protein sequence identity to *Haemophilus influenzae*, the type species of the genus, was 77%, demonstrating that [*Haemophilus*] *haemoglobinophilus* is not properly classified as a member of the genus *Haemophilus*. Based on the phylogenetic comparisons, the taxa [*Haemophilus*] *haemoglobinophilus* and taxon 35 are proposed to be included with a new genus *Canicola* with one species, *Canicola haemoglobinophilus* which is reclassified from [*Haemophilus*] *haemoglobinophilus.* Phenotypic characters obtained with isolates genetically approved to represent *Canicola haemoglobinophilus* were in accordance with those of *Pasteurellaceae,* and the new genus can be separated from most of the existing genera by a positive catalase reaction, lack of V-factor requirement for growth, lack of haemolysis of blood agar, and negative Voges-Proskauer and urease tests. The new genus cannot be separated by biochemical and physiological characteristics alone from the genera *Aggregatibacter, Avibacterium, Frederiksenia* and *Spirabiliibacterium.* However, MALDI-TOF mass spectroscopy and also RpoB amino acid signatures allowed a clear separation from these taxa, supporting the existence of a new genus. The GC mole % is 37.0-37.8 for the genus based on the whole genomic sequences. The type strain of *Canicola haemoglobinophilus* is CCUG 3714^{T} (= ATCC 19416^{T} = NCTC 1659^{T}) isolated in 1901 from the prepuce of a dog in Germany.

Pasteurella sensu stricto [1] and other taxa of *Pasteurellaceae* obtained from lesions in dogs or wounds inflicted by dogs represent a diagnostic enigma if based upon phenotypic characterization [2]. However, Dousse *et al.* [3] analyzed 40 type- and reference strains and 267 isolates from routine diagnostic cases by 16S rRNA and *rpoB* gene sequencing for unambiguous species determination before submitting them to

phenotypic identification, which allowed proper identification of 240 out of 267 field isolates (90%). The genus *Frederiksenia* including almost exclusively dog isolates was reclassified from taxon 16 of Bisgaard which has improved the identification of this taxon [4]. However, other taxa, like biovar U24 of Biberstein [5] associated with respiratory – and urogenital tract infections in dogs and [*Haemophilus*] *haemoglobinophilus*/"*H. canis*" [6], have remained unclassified and unreported. In the present paper, we compare these taxa with taxon 35, a previously unreported group of *Pasteurellaceae* from dogs, to improve our understanding and the diagnosis of these organisms. The insertion of the genus name in brackets signifies misclassification of the species with respect to the type species of the genus (*Haemophilus influenzae*) [7, 8]. Taxon 35 of Bisgaard has remained unpublished, however, it was described in a conference proceeding report [9]**.** Taxon 35 of Bisgaard was classified by a unique phenotypic profile related to a negative oxidase reaction, acid formation from (-)-D-fructose and (-)-D-mannitol and a positive α-fucosidase test separating it from [*Haemophilus*] *haemoglobinophilus* [9]. [*Haemophilus*] *haemoglobinophilus* was originally isolated from suppurative inflammation of the prepuce of dogs [6, 10]. Friedberger originally labelled his strain as XIII^{T}, and his strain has become the type strain of the species. Kilian [6] characterized five strains including the type strain. Rivers [11] reported on the characteristics of American strains. While Rivers [11] found his strains (-)-D-fructose and (+)-D-galactose positive, Kilian found his strains negative in the same characters. Biberstein *et al.* [5] reported on 356 animal isolates of urease negative and indole positive strains of *Pasteurellaceae,* 93 isolates (26%) of which were unassignable with known species. These isolates made up 24 biovars, eleven of which were obtained from dogs. The most prevalent biovar (U24) made up 24 of the 37 dog isolates. The U24 biovar was subsequently shown to belong to Bisgaard taxon 35 (Bisgaard, unpublished data). Because taxon 35 might represent atypical isolates of *Pasteurella multocida* from dogs, some isolates were included in a *Hpa*II ribotype comparison reported for *P. multocida* [12]. These investigations showed that taxon 35 diverged from *P. multocida* (supplementary Table 1). Other strains classified as EF group 32 have also been proposed to belong to taxon 35 of Bisgaard (https://www.ccug.se/).

In the current study, 21 strains were investigated including the type strain of [*Haemophilus*] *haemoglobinophilus* (supplementary Table 1). Six strains from the investigation of Biberstein *et al.* [5] representing biotype U24 were included.

Searches for sequences in public databases were performed by BLAST [13]. Determination of pairwise similarity was performed by the WATER program of EMBOSS [14]. Multiple alignments and neighbour joining phylogenetic analysis including calculation of bootstrap support were done by ClustalX2 [15], and MEGA7 [16] and used for graphical representation of trees.

Sequencing of the 16S rRNA gene was performed according to previous reports [17, 18]. The 16S rRNA gene sequence reference was chosen from the type strain (NCTC 1659^{T}) of the fully closed genome (accession number UGHF01). Among the six operons, two 16S rRNA gene sequence types were found with only 2 nt differences between the types. Slight variation was found for the three 16S rRNA gene sequences published previously for the type strain (supplementary Table 1) with between 2 and 8 nt differences. A monophyletic group was formed by strains of [*Haemophilus*] *haemoglobinophilus* and taxon 35 of Bisgaard based on 16S rRNA gene sequence comparison (supplementary Fig. 1). The lowest similarity within the group was 97.8% between strains CCUG 47869 and A478/88 which documented that taxon 35 of Bisgaard was closely related to [*Haemophilus*] *haemoglobinophilus* at the species level using 97% as a threshold for species level separation [19].

16S rRNA gene sequence relationships at the species level was investigated by EzBioCloud [20] and showed that *Glaesserella australis* was closest related to [*Haemophilus*] *haemoglobinophilus* with 96.1%. This is higher than the 95% 16S rRNA gene sequence similarity separating most genera of *Pasteurellaceae* [8]. In the past, DeLey et al. [21] allocated [*Haemophilus*] *haemoglobinophilus* with the *Pasteurella multocida* rRNA branch while Dewhirst et al. [22] allocated [*Haemophilus*] *haemoglobinophilus* with cluster 3C although with a divergent position compared to

other species of that cluster. The strains NCTC 8540 and NCTC 10619 were reported with phenotypic characteristics in Kilian [6] and the whole genomic sequenced with acc. no. UGHJ01 and UGHE01, respectively. The 16S rRNA gene sequence similarity was 99.87% between the type strain and strains NCTC 8540 and NCTC 10619 based on the published whole genomic sequences related to only 2 or 3 nt differences. The 16S rRNA gene sequence operons of the strains were identical in triplets and three operons sequenced from NCTC 8540 were identical to three in NCTC 10619. Between the two types of operons two nt differences were found. Strain CCUG 16472 was only represented by 60% coverage of the 16S rRNA gene sequence related to Illumina sequencing of the strain and it was not included with the phylogenetic comparison. In the region available for comparison, the similarity was 98.29% to the type strain.

Classification based on *rpoB* gene sequence comparison has previously been used with *Pasteurellaceae* [22].The partial *rpoB* sequences of all isolates (supplementary Table 1) were determined according to Korczak *et al*. [23] and Mollet *et al*. [24], covering the region 509-680 (*Escherichia coli* pos.) of the deduced protein sequence. The *rpoB* gene sequences were extracted from the whole genomic sequences from strains NCTC 1659^{T} and CCUG 16472. A monophyletic group was formed based on partial *rpoB* gene sequence comparison of strains of taxon 35 and [*Haemophilus*] *haemoglobinophilus* (supplementary Fig. 2). The lowest similarity within the group was 96.5%. The highest *rpoB* gene sequence similarity outside the group was 85.7% to *Caviibacterium pharyngocola* (strain 7.3 acc. no. AY314039). These results indicate that taxon 35 and [*Haemophilus*] *haemoglobinophilus* are forming one species since most species of *Pasteurellaceae* are separated by 95% *rpoB* gene sequence similarity [8]. The relationship at genus level is above the level of 85% *rpoB* gene sequence similarity separating most genera of *Pasteurellaceae* [8].

The whole genomic sequence was generated for strain CCUG 16472 representing taxon 35 by IlluminaHiseq2000 (BGI technology) and assembled by Geneious ver. 6 which resulted in 109 contigs. The GC mole content determined from the whole genomic sequence was 37.1% for strain CCUG 16472 and the genome size 2.88 Mb

which is slightly higher than that of the type strain with a genome size of 2.42 Mb. RAST (Rapid Annotation using Subsystem Technology) [25] analysis showed that the larger genome size of strain CCUG 16472 was reflected in more proteins predicted in almost half of the subsystem categories compared to strain NCTC 1659^{T}. A similar genome size variation has been observed for other bacteria. For closed genomes of *E. coli* at NCBI [26], the GC mol % vary only from 50.2 to 51.6, however, the genome sizes cover an interval from 4.0 to 6.2 Mb.

The Average nucleotide identity (ANI) [27] was 94.72% between the genome of NCTC 1659^{T} and CCUG 16472 indicating that the strains belong to the same species [28] although the majority of strains within the same species were separated by more than 96% ANI when all genomes of GenBank were compared [28]. For strains NCTC 8540 and NCTC 10619, ANI was 98.66 and 98.83% respectively to the type strain, documenting close relatedness at the species level. DNA-DNA reassociation (DDR) was estimated to 57.2% by Genome to Genome Distance calculator (GGDC) [29, 30] between NCTC 1659^{T} and CCUG 16472. The GGDC estimate is below the 70% being the conventional threshold for species level separation [31]. A similar lower DDR compared to ANI has been reported for the relationship between cryptic clade I of *E. coli* and traditional *E. coli* [32]. In that study cryptic clade I was interpreted as a subspecies of *E. coli*. With the same hosts and lesion types reported as well as lack of phenotypic divergence between subgroups, all strains of [*Haemophilus*] *haemoglobinophilus* and taxon 35 of Bisgaard were allocated to the same species.

Concatenated conserved protein sequence phylogenies were recently published [33, 34] and here [*Haemophilus*] *haemoglobinophilus* formed a monophyletic unit with *Bisgaardia hudsonensis* supported by 94% bootstrap. The highest conserved protein sequence identity of 81% was obtained between [*Haemophilus*] *haemoglobinophilus* and genomospecies 1 of *Rodentibacter*. The highest protein sequence identity between genera in that comparison was 85% documenting a genus like position of [*Haemophilus*] *haemoglobinophilus*.

Kuhnert & Korczak [39] described the use of *recN* gene sequences for estimating whole-genome sequence similarity. For strains NCTC 1659^T and CCUG 16472, the *recN* gene sequences were extracted from the whole genomic sequence. In addition four strains were selected for *recN* sequencing based on the *rpoB* and 16S rRNA gene sequence comparisons (supplementary Table 1). PCR amplification of the *recN* gene sequence was performed by 478 RL and firstRL [35]. Internal primers for sequencing were forward recNtx35intF 5'-GGAGCAACGTATGGGACAA and reverse recNtx35infF 5'-TATGCCCACAGAATTGATCG with 1311 bp of the gene being sequenced in five strains. Phylogenetic analysis confirmed the monophyletic relationship between the strains (supplementary Fig. 3). Down to 93.7% *recN* similarity was found (NCTC 1659^T, CCUG 16472) between the six strains selected to represent [*Haemophilus*] *haemoglobinophilus* and taxon 35. Outside the [*Haemophilus*] *haemoglobinophilus*/taxon 35 complex, the highest similarity of 83-84% was demonstrated with the type strain of *Histophilus somni*. When these values were converted to whole genome similarity values according to Ziegler [36], they showed 81% similarity within the group and 57% to the closest related species, *Histophilus somni*. The within group genome similarity is in accordance with the variation observed on species level for *Pasteurellaceae* [8, 33], whereas the similarity to the closest related genus, *Histophilus somni* (57%) was somewhat higher than the average genus level boundary of 40% observed for *Pasteurellaceae* [8, 33]. Compared to ANI and GGDC estimation of DDR, the 81% DDR estimated based on *recN* indicated a closer relatedness between members of the species. The divergence seems related to the use of only one gene (*recN*) for the prediction of DDR whereas ANI and GGDC are based on most of the whole genomic sequences.

The group of [*Haemophilus*] *haemoglobinophilus*/taxon 35 strains diverged at the genus level from other genera of *Pasteurellaceae* with respect to *rpoB*, 16S rRNA, *recN* and whole genomic similarities. It is proposed that [*Haemophilus*] *haemoglobinophilus* and taxon 35 of Bisgaard are reclassified as a new genus *Canicola haemoglobinophilus*. The present investigation confirmed the association of *Canicola haemogloginophilus*

with dogs and that this taxon has been underreported due to difficulties in obtaining an unambiguous diagnosis based upon classical phenotypical characters.

Annotation by RAST predicted three syntenic homologs, A, B and C of the cytolethal distending proteins (Cdt) in the genomes of both the type strain NCTC 1659^T and CCUG 16472. Comparison at the level of protein sequence showed 72, 84 and 70% identity between the type strain of [*Haemophilus*] *haemoglobinophilus* and CCUG 16472 for the three homologs CdtA, CdtB and CdtC, respectively. The CdtA homolog was found with 99% identity to the type strain in both strain NCTC 8540 and NCTC 10619 for which whole genomic sequences have been determined. For both strains, the CdtB and CdtC homologs were identical to the type strain. Comparative analysis showed the closest relationship to *Frederiksenia canicola* with identities of 68, 75 and 50% for CdtA, CdtB and CdtC, respectively. The homologs have also been found in [*Haemophilus*] *ducreyi*, *Aggregatibacter actinomycetemcomitans*, *Glaesserella parasuis* and *Avibacterium paragallinarum* of the *Pasteurellaceae* [37, 38]. CdtA, CdtB and CdtC are known to form a tripartite complex required for the Cdt activity. Cdt can induce G2/M cell cycle arrest, chromatin fragmentation, cell distention and nucleus enlargement [39]. Outside members of *Pasteurellaceae*, Cdt is best known from *Escherichia coli*, *Salmonella typhi*, *Campylobacter jejuni* and *Helicobacter hepaticus* [40]. Further work including the development of a PCR test is needed to show if the presence of the *cdt* gene sequence and its diversity can be used to separate *Canicola haemoglobinophilus* from other canine taxa of *Pasteurellaceae* including *Frederiksenia* and *Pasteurella sensu stricto*.

Strains were characterized phenotypically according to Bisgaard *et al.* [41] as further explained in Christensen *et al*. [8]. Phenotypic characters obtained with isolates of taxon 35 genetically approved to represent [*Haemophilus*] *haemoglobinophilus* were in accordance with those of *Pasteurellaceae* [33] and "*Bacillus haemoglobinophilus canis*" as reported by Rivers [11]. However, differences in acid production from (-)-D-fructose and α-fucosidase separate the strains investigated from those reported by Kilian [6]. The new genus can be separated from the other genera by a positive catalase reaction,

lack of growth factor requirements for V-factor, lack of haemolysis of blood agar, negative Voges-Proskauer and negative urease tests (supplementary Table 2). It is also shown in this table that *Canicola haemoglobinophilus* cannot be separated from the genera *Aggregatibacter, Avibacterium, Frederiksenia* and *Spirabiliibacterium*. Among these genera only *Frederikseria* includes bacteria associated with dogs. Differences in α-glucosidase (PNPG) and α-fucosidase (ONPF) separate the *Canicola haemoglobinophilus* from *Frederiksenia*, however, the PNPG reaction was weak for members of the genus and only three stains were tested and found negative for the ONPF reaction [42].

The type strain of [*Haemophilus*] *haemoglobinophilus* was found with haemin requirement (negative porphyrin test) whereas some other strains were found without this requirement. We therefore analysed the four genomes published for [*Haemophilus*] *haemoglobinophilus* and taxon 35 and found all proteins of the biosynthesis pathway described in Harris et al. [43] present in strain CCUG 16472 with high coverage (90-100%) and identity (62-91%). However, in the type strain of [*Haemophilus*] *haemoglobinophilus* and in strains NCTC 10619 and NCTC 8540 only the proteins HemA, HemD and HemH could be predicted with similar ranges of coverage and identity whereas HemB, HemC, HemE, HemL and HemN showed coverages/identities of 37/62, 11/78, 27/84, 9/66 and 52%/28% respectively (HemG not significant) to the proteins predicted in *Haemophilus parainfluenzae* strain T3T1 used as a reference in Harris et al. [43]. These results confirm the variable nature of this property in the new genus proposed.

The fatty acid composition was in line with other genera of *Pasteurellaceae* with a dominance of $C_{12:0}$, $C_{12:0\ ALDE}$, $C_{14:0}$, $C_{14:0}$ 3OH/$C_{16:1}$ ISOI, $C_{16:1}$ ω7c and $C_{16:0}$. The proportion of the fatty acids $C_{12:0}$ and $C_{12:0\ ALDE}$ in *Canicola haemoglobinophilus* was higher than in other genera of *Pasteurellaceae* (supplementary Table 3).

For MALDI-TOF MS analysis the isolates were grown on trypticase soy agar plates with 5% sheep blood or on chocolate agar plates (Becton Dickinson, Allschwil, Switzerland)

at 37°C for up to 24 h. The MALDI-TOF MS analysis was done as described previously [44]. In short, a single colony was then taken with a toothpick, smeared on a steel plate and overlaid with 1 µl of HCCA (alpha-cyano-4-hydroxycinnamic acid) matrix solution. After air drying, the samples were measured using standard settings in the “Flex control” software. Identification was done with Biotyper 3.0 commercial database in combination with the *Pasteurellaceae* project database established previously [45]. MALDI-TOF mass spectrometry separated *Canicola haemoglobinophilus* from other taxa of *Pasteurellaceae*. All strains of *Canicola haemoglobinophilus* were identified by scores ≥ 2 to the type strain of [*Haemophilus*] *haemoglobinophilus* and clearly separated from any other species by scores ≤ 1.8 (supplementary Table 4).

RpoB signature sequences separated *Canicola* from the other genera of *Pasteurellaceae* by P566L (data not shown), Q798N, Q1020K, S1060N and Q1245K which included *Aggregatibacter, Avibacterium, Frederiksenia* and *Spirabiliibacterium* (supplementary Table 5).

Description of *Canicola* gen. nov.

Canicola (ca.ni’co.la. L. masc. n. *canis* dog; L. suff. *-cola* (from L. masc. n. *incola*) dweller, inhabitant; N.L. masc. n. *Canicola* a dweller of dogs).

The description of the genus extends the phenotypical properties reported by Kilian [6] for [*Haemophilus*] *haemoglobinophilus* based on five strains characterized. However, negative reactions in acid formation from (-)-D-fructose and α-fucosidase were not confirmed. The present findings, however, confirmed the description of Rivers [7]. After 24 hours of aerobic incubation on bovine blood agar, the size of the colonies vary from pinpoint to 1 mm in diameter. Colonies are circular, regular, slightly elevated, smooth and grayish with an entire margin. Colonies might be surrounded by a greenish discolouration. An unguent-like consistency is observed, and colonies do not adhere to the agar surface. The bacteria appear as short rods or pleomorphic cells, stain Gram-negative, and do not demonstrate motility. Members of the genus are catalase positive, but variable in the oxidase test and in haemin requirement (porphyrin test). V-factor is not required for growth. A fermentative reaction is observed with (+)-D-glucose in Hugh

& Leifsons medium. Nitrate is reduced without gas formation. The alanine aminopeptidase test is positive. Variable reactions are observed for ornithine decarboxylase, indole and phosphatase (most strains positive) (see supplementary Table 2). Negative reactions are observed for Simmons citrate, acid from mucate, base from malonate, H_2S/TSI, growth in KCN, methyl red, Voges-Proskauer, urease, arginine dihydrolase, lysine decarboxylase, phenylalanine deaminase, gelatinase, hydrolysis of Tween 20 and 80, growth on MacConkey agar, and formation of pigment. Acid is produced from (-)-D-ribose, (-)-D-fructose, (+)-D-glucose, and sucrose. Variable reactions as to acid production are observed with glycerol, (+)-D-xylose, *meso*-inositol, (-)-D-mannitol, (-)-L-fucose, (+)-D-galactose, (+)-D-mannose, lactose, maltose, trehalose, raffinose, and dextrin, while acid is not produced from *meso*-erythriol, adonitol, (+)-D-arabitol, xylitol, (+)-L- arabinose, (-)-L-xylose, dulcitol, (-)-D-sorbitol, (+)-D-fucose, (+)-L-rhamnose, (-)-L-sorbose, cellobiose, (+)-D-melibiose, (+)-D-melezitose, (+)-D-glycogen, inulin, esculin, amygdalin, arbutin, gentiobiose, salicin, and (+)-D-turanose. Gas is not produced from (+)-D-glucose. The test for α-fucosidase (ONPF) is positive whereas the reactions of α–galactosidase, α-mannosidase, β-glucosidase (NPG), β-glucoronidase (PGUA) and β-xylosidase (ONPX) are negative. Reactions observed in β-galactosidase (ONPG) and α-glucosidase (PNPG) are variable for strains of the genus. The fatty acid composition is in line with other genera of *Pasteurellaceae* with a dominance of $C_{12:0}$, $C_{12:0\ ALDE}$, $C_{14:0}$, $C_{14:0\ 3OH/C_{16:1}\ ISO\ I}$, $C_{16:1\ \omega7c}$ and $C_{16:0}$. Members of this new genus are phenotypically very diverse and difficult to separate from other genera of *Pasteurellaceae*. The GC mole content was 37.0-37.8% determined from whole genomic sequences. The type species is *Canicola haemoglobinophilus.*

Description of *Canicola haemoglobinophilus* comb. nov.

hae.mo.glo.bi.no'phi.lus. N.L. neut. n. *haemoglobinum*, hemoglobin; N.L. masc. adj. *philus* (from Gr. masc. adj. *philos*), friend, loving; N.L. masc. adj. *haemoglobinophilus*, hemoglobin-loving.

The properties of the species is according to the description of the genus with the following emendments. The requirement of haemin for growth is variable, and the type

strain requires haemin. Different reactions are observed for acid production from *meso*-inositol, (+)-D-galactose, lactose, trehalose and raffinose, however, the type strain tests positive in these characters. The type strain is found oxidase positive, indole positive and ornithine decarboxylase and phosphatase negative. The type strain is negative for the α-fucosidase test. The type strain CCUG 3714^T (= ATCC 19416^T = NCTC 1659^T), was isolated from the prepuce of a dog in Germany in 1901.

Basonym: *Haemophilus haemoglobinophilus* (Lehmann and Neumann 1907) Murray 1939 (Approved Lists 1980).

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Ethical statement

Samples were obtained from dead animals only and thus complied with international ethical guidelines.

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Legend for figures

Fig. 1. Phylogenetic relationships between the type strain of *Canicola* ([*Haemophilus*]) *haemoglobinophilus* and existing genera of *Pasteurellaceae* based on neighbour-joining analysis of near full length 16S rRNA gene sequences. Supports for monophyletic groups by bootstrap-analysis are indicated as numbers out of 100. The strains are followed by DDBJ/EMBL/GenBank accession numbers in parenthesis. The scale bar represents sequence variation considering the evolutionary model of Jukes & Cantor and Neighbour-Joining algorithm used to construct the phylogenetic tree [46, 47].