Multilocus sequence phylogenetic analysis of *Avibacterium*

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This study examined 49 field isolates of the genus *Avibacterium*, with the 49 being allocated to 36 epidemiologically unrelated groups and one isolate from each group being examined in detail. In addition, six type and reference strains were investigated. Phylogenetic analysis of partially sequenced *recN*, *rpoB*, *infB*, *pgi* and *sodA* genes confirmed the existence of the species *Avibacterium paragallinarum*, while a species complex encompassing *Avibacterium volantium*, *Avibacterium avium*, *Avibacterium gallinarum*, *Avibacterium endocarditis* and *Avibacterium* sp. A could not be resolved. All isolates shared at least one identical sequence in one gene, indicating low diversity or horizontal gene transfer (HGT) between isolates. Such HGT between isolates of defined species and unclassified isolates combined with high sequence similarity can be explained as the result of an ongoing speciation process. The alternative explanation is that *Av. volantium*, *Av. avium* and *Avibacterium* sp. A were misclassified originally. Except for *Av. paragallinarum*, identification of species of *Avibacterium* seems problematic, even by DNA sequencing, as shown in the present investigation. The results indicate that *Avibacterium* probably contains only two or three species. Until the taxonomic revision is completed we recommend that isolates that do not fit with named species by genotype and phenotype be designated *Avibacterium* sp.

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INTRODUCTION

Reclassification of *Pasteurella gallinarum* (Hall *et al.*, 1955) in the new genus *Avibacterium* (Blackall *et al.*, 2005) also included the taxa *Pasteurella avium*, *Pasteurella volantium* and *Pasteurella* sp. A, originally described by Mutters *et al.* (1985), and [*Haemophilus*] *paragallinarum* (Biberstein & White, 1969). Subsequently, a new species, *Avibacterium endocarditis*, has been reported by Bisgaard *et al.* (2007). All members have been isolated from birds, in which they probably occur as opportunistic pathogens, with the exception of *Avibacterium paragallinarum*, which is considered a primary pathogen (Blackall, 1999; Welchman *et al.*, 2010). Some members of *Avibacterium* are difficult to

cultivate, and phenotypic identification at species level is problematic for some isolates due to variable species characteristics (Blackall & Nørskov-Lauritsen, 2008). The description of the species *Avibacterium avium* was only based on three isolates (Mutters *et al.*, 1985), increasing the risk of the existence of field isolates which do not fit into the species description (Christensen *et al.*, 2007). Dewhirst *et al.* (1993) documented that *Avibacterium* was monophyletic (16S rRNA cluster 3A); however, later phylogenetic analysis showed polyphyly of *Avibacterium gallinarum* (Blackall *et al.*, 2005) just as major difficulties were experienced in resolving the phylogeny of other members of the genus *Avibacterium* (Christensen *et al.*, 2009). In addition, phenotypic variants of taxa of *Avibacterium* that did not fit into the formal species descriptions have been recognized for some time (Blackall, 1988).

Multilocus sequence analysis (MLSA) has been suggested as an alternative to 16S rRNA gene analysis and recommended for improving identification at the species level of

Abbreviations: ANI, average nucleotide identity; DDN, DNA–DNA renaturation; ERIC, enterobacterial repetitive intergenic consensus; HGT, horizontal gene transfer; MLSA, multilocus sequence analysis.

A supplementary figure and five supplementary tables, with supplementary references, are available with the online version of this paper.

members of the *Pasteurellaceae* (Bisgaard *et al.*, 2010; Christensen & Bisgaard, 2010). For MLSA we chose partial sequences of the genes *recN*, *rpoB*, *infB*, *pgi* and *sodA*. Next to 16S rRNA the most frequent gene used for single-gene sequence analysis of members of *Pasteurellaceae* has been *rpoB*, encoding the β -subunit of DNA-dependent RNA polymerase (Christensen & Bisgaard, 2010). The other genes selected included *recN*, encoding a DNA-dependent repair protein, *infB*, encoding translation initiation factor 2, *sodA*, encoding manganese-dependent superoxide dismutase, and *pgi*, encoding phosphoglucose isomerase, all of which have been used in previous studies of the classification and phylogeny of members of *Pasteurellaceae* (Nørskov-Lauritsen *et al.*, 2004, 2005; Gautier *et al.*, 2005; Kuhnert & Korczak, 2006).

Another way to analyse such data is by MLST. In this technique, every sequence difference is scored as an allele, and further comparison is based on these allele differences. The idea behind both MLSA and MLST is to base separation of clones and populations of taxa on slowly evolving genes which are neutral in regard to selection (Maiden *et al.*, 1998). Although horizontal gene transfer (HGT) will not compromise MLST analysis between different populations of the same species, it will lead to wrong conclusions if genes are transferred between species. Little information is available as to the frequency and impact of horizontal transfer of conserved genes within *Avibacterium*. For the same reasons, the aims of this investigation were to analyse members of *Avibacterium* by MLSA and MLST in order to improve and clarify classification of a number of unclassified isolates and of isolates which in some aspects are related to validly published species, and to investigate whether indications of HGT exist for the genes investigated. A preliminary report of the investigation has been published (Bisgaard *et al.*, 2011).

METHODS

Isolates analysed and phenotypic characteristics. The 55 isolates analysed represented 49 *Avibacterium* field isolates which were compared with six type and reference strains (Supplementary Table S1). Phenotypes were analysed according to Christensen *et al.* (2007).

DNA sequencing. For *recN* gene sequencing, the primers *recN*_{first-L} (5'-ATGCTTANYCAWCTYACKATYAATMATTTC-3') and *recN*_{first-R} (5'-CCRCTAATYCCMACATCNACYTCATC-3') were used for amplification of 1.4 kb of the gene at 50 °C annealing temperature according to Kuhnert & Korczak (2006). The PCR product was sequenced with the same primers to obtain a partial gene sequence (positions 528–879; positions refer to accession number AE004439 of strain pm70) (Kuhnert & Korczak, 2006). To obtain nearly full-length sequences (positions 95–1337; positions refer to accession number AE004439 of *Pasteurella multocida* strain pm70) from 11 isolates, the two internal primers *recN*-1 (5'-GTAAGTATTGATGCGTGCTG-3') and *recN*-2 (5'-CAGCAGCGCATCAATACTTAC-3') were also used. The *recN* gene has proved useful in delineating genetic relatedness of isolates and supplements DNA–DNA hybridization data (Kuhnert & Korczak, 2006). Genome similarities were calculated using the formula in Zeigler (2003), as described by Kuhnert & Korczak (2006). The partial *rpoB* sequences were determined according to Mollet *et al.* (1997),

covering positions 1598–1967 (positions refer to AE004439 of strain pm70) as reported previously (Angen *et al.*, 2003; Korczak *et al.*, 2004). In addition to published primers, the forward PCR primer *rpoB*_{pm} (5'-GCAGTGAAAGAATTCTTTGGTTC-3') was used for PCR amplification and DNA sequencing with some isolates. The partial *infB* gene sequence was generated (positions 509–1050; positions refer to accession number AE004439 of strain pm70) according to the methods of Kuhnert *et al.* (2004) and Mayor *et al.* (2006). The partial *pgi* sequence (positions 450–749; positions refer to accession number AE004439 of strain pm70) was PCR-amplified and sequenced using the primers *Pgi*838F and *Pgi*1331R as described by Nørskov-Lauritsen *et al.* (2005). The partial *sodA* gene (positions 103–467; positions refer to accession number AE004439 of strain pm70) was PCR-amplified and sequenced according to Gautier *et al.* (2005). To allow comparison with another genus of the *Pasteurellaceae*, all type strains of *Mannheimia* were compared with respect to the same genes analysed in *Avibacterium* (Supplementary Table S2).

PCR amplification of the partial *recA* gene sequence and subsequent DNA sequencing were performed according to the method of Nørskov-Lauritsen *et al.* (2005). In addition to primers included in that publication, the primers *recA*54fav (5'-GAAAARCAATTTG-GKAAAGGY-3') and *recA*617rav (5'-TTACCTACATHACVCC-AAT-3') were used to achieve PCR amplification.

Enterobacterial repetitive intergenic consensus (ERIC)-PCR of Dutch isolates was performed according to the method of Soriano *et al.* (2004).

Phylogenetic analysis. Multiple alignment and neighbour-joining phylogenetic analysis were performed using CLUSTAL_X, including bootstrap resampling with 100 replications (Thompson *et al.*, 1997) and phylogenetic trees constructed using MEGA4 (Tamura *et al.*, 2007). Majority rule consensus comparison between phylogenies was performed using CONSENSE in the PHYLIP package (Felsenstein, 1995).

DNA similarity based on whole genomic sequences. Pairwise comparisons between single genes were performed using the program WATER included in EMBOSS (Rice *et al.*, 2000).

DNA–DNA hybridizations of type strains of *Av. gallinarum*, *Av. avium* and *Avibacterium volantium* were performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig), as described by De Ley *et al.* (1970) and taking into consideration the modifications described by Huß *et al.* (1983), using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier thermostat-fitted 6 × 6 multicell charger and a temperature controller with *in situ* temperature probe (Varian). Measurements were performed in 2 × SSC at 66 °C in duplicate. The whole genomic sequences of the type strains of *Av. gallinarum*, *Av. paragallinarum* and *Av. endocarditidis* were included for comparison. Draft genomes were obtained by the 454 paired-ends technology (CD Genomics, GATC Biotech). The genomes were not fully assembled. Calculation of average nucleotide identity (ANI) based on the whole genomic sequences of the three type strains was according to the method of Goris *et al.* (2007), performed on a desktop computer with Windows XP by use of standalone BLAST (Altschul *et al.*, 1997) downloaded from NCBI, EMBOSS (Rice *et al.*, 2000) and Office Excel 2003 (Microsoft).

Analysis of clonality and HGT. Analysis for linkage disequilibrium was carried out according to the method of Haubold & Hudson (2000). Hypotheses about gene transfer between isolates of *Avibacterium* were tested by Simplot and PLATO (Grassly & Holmes, 1997; Lole *et al.*, 1999). In these analyses the sequences of the five partially sequenced genes were concatenated to 1840 nt. Finally, sequences were evaluated by scoring all differences between homologous sequences as different alleles and combining allele types of the five genes into a sequence type. The dataset was then used to

construct a minimum-spanning tree using the program from the PubMLST server (<http://pubmlst.org/perl/mlstanalyse/mlstanalyse.pl?site=pubmlst&page=mst&referer=pubmlst.org>).

Identification of *Av. paragallinarum* by PCR. Fourteen strains (see Supplementary Table S1) were tested, including those identified as *Av. paragallinarum* by phenotypic identification as well as isolates found to be closely related to the type strain of *Av. paragallinarum* by phylogenetic analysis. The PCR was performed as described by Chen *et al.* (1996) by using DNA template extracted with a DNeasy Blood & Tissue kit (Qiagen).

RESULTS

Phenotypic characteristics

With the exception of 10 isolates, phenotypic characters allowed classification with known taxa of *Avibacterium*. Three isolates (RB1554, RB1740 and RB1857) classified as *Av. volantium*-like deviated in D-(+)-arabitol and V-factor requirement from this species, while a fourth isolate (RB410) only deviated in V-factor requirement. Three isolates (B69/96/3, B70/96/1 and CCUG 18407) classified as *Avibacterium* sp. differed from *Av. gallinarum* in maltose and dextrin, while a single isolate (CCUG 1208) differed from *Avibacterium* sp. A in V-factor requirement and acid production from D-(+)-arabitol and dextrin. Isolate P. sp. pp. 23 differed from the type strain of *Av. gallinarum* in being positive for acid formation from D-(−)-sorbitol. Isolate 55000 was late positive in acid formation from xylitol and without acid formation from trehalose compared with the type strain of *Av. endocarditidis*. In addition, isolate 55000 was negative in α -glucosidase formation compared with the type strain of *Av. endocarditidis*. Three isolates classified with taxon 49 (Supplementary Table S1) shared a phenotype with *Av. volantium*. The separate classification is used to designate isolation from pheasants compared with the original description from chicken.

DNA sequencing

For the five genes *rpoB*, *pgi*, *recN*, *sodA* and *infB*, sequences were obtained of 370, 300, 352, 362 and 456 nt, respectively, from the 49 isolates analysed in addition to reference strains. Some reference strains had already been sequenced and deposited with the nucleotide database. For 10 epidemiologically related isolates, identity was initially achieved with the *rpoB* sequences and only one isolate (1248) was selected for further characterization (Supplementary Table S1). Isolate SP2009-1302 represented five isolates (including SP2009-1397, SP2009-1354, SP2009-1174 and SP2009-1353) isolated from coryza of chickens, demonstrating the same ERIC-PCR profile and *rpoB* sequence. Isolate SP2009-508 represented two isolates (the other being SP2009-740) which also shared an ERIC-PCR profile and *rpoB* sequence. Biochemically (NAD, oxidase, catalase, D-galactose, maltose, D-mannitol and trehalose) these two isolates reacted the same as the other five isolates, and clinically all seven produced 'coryza'-like symptoms. Isolates 05-2288 and

05-2290 originated from different chickens on the same farm and in the same house, and 05-2291 and 05-2292 originated from the same farm but different houses, whereas isolate 05-2286 represented a third farm. As a consequence, 05-2288 and 05-2290 are considered epidemiologically related as well as 05-2291 and 05-2292. Single isolates from different organs (liver, spleen) of the same animal always had the same sequence, and all epidemiologically related isolates always had the same sequence (Supplementary Table S1). This left 36 epidemiologically unrelated isolates for analysis, and counting identical sequences in all five gene sequences resulted in 35 different sequence types, including type and reference strains (Supplementary Table S1).

Three different versions of *recA* gene sequences were found in the *Av. gallinarum* genome. Similarities of translated sequences showed 99.2 % similarity between two and 93.4 % to the third homologue in those genomes. The most divergent of the three *recA* copies was unrelated to members of the genus *Avibacterium* (BLASTP in GenBank and Swiss-Prot). The type strain of *Av. gallinarum*, in addition to 12 other isolates (G915/84, CCUG 18361, CCUG 18395, CCUG 18401, CCUG 18403, CCUG 18404, CCUG 18406, PG145, 46671, 47483/5, 05-2290liver and P291), showed double bands in the initial PCR. For two isolates (84611 and 1248), 10 double peaks in sequences were observed, which did not allow identification of nucleotides. It was assumed that these problems were caused by multiple copies of the *recA* gene in some members of the genus *Avibacterium*, and the gene was therefore excluded from further analysis.

Phylogenetic analysis

Phylogenies were constructed for each of the five genes (Fig. 1a–e). Bootstrap support higher than 50 % was used to define a group. The type strain of *Av. gallinarum* was closely related to isolates CCUG 1208 and RB410 with respect to three genes, but not for *rpoB* and *infB*. The *rpoB* phylogeny further showed a relationship between isolate CCUG 18404 and the type strain of *Av. gallinarum*. With respect to *infB*, isolate CCUG 1208 was related to B69/96/3 and B70/96/1, whereas the type strain of *Av. gallinarum* was related to isolates P. sp. pp. 6, CCUG 18395 and RB410. In addition to the above isolates CCUG 1208 and RB410, the *recN* phylogenetic comparison linked SP2009-508, P291, CCUG 18404, 05-2286spleen, 05-2288liver, CCUG 18407 and CCUG 18401 to the type strain of *Av. gallinarum*. The *pgi* phylogenetic comparison linked the type strain with CCUG 18404, P. sp. pp. 23, 05-2286spleen, 05-2288liver, 05-2291, RB1857, RB1554, RB1740, CCUG 19361, CCUG 18395, B69/96/3, B70/96/1, CCUG 18407 and CCUG 18401 in addition to isolates CCUG 1208 and RB410 (isolates with identical sequences are listed in Supplementary Table S1).

The type strain of *Av. volantium* always formed a monophyletic group with isolate 47483/5, and the two isolates even demonstrated identical *recN*, *sodA* and *infB* gene sequences (Supplementary Table S1). For *recN*, a relationship was further found between the type strain of *Av.*

Fig. 1**(a) *recN*** **(b) *rpoB***

volantium and isolates 1248, B11/99/2 and 46671, while a relationship was found with 84611 for *infB* (Fig. 1a–e). The type strain of *Av. avium* was related to the reference strain of *Avibacterium* sp. A with respect to *rpoB* and *sodA*, but not for the other genes. A high bootstrap value for *pgi* linked the type strains of *Av. avium* and *Av. endocarditidis*. The *recN* phylogenetic comparison showed a relationship between the type strain of *Av. avium* and isolates CCUG 18406, CCUG 18403, G915/84, P. sp. pp. 23 and CCUG 19361 (Fig. 1a–e). The type strain of *Av. endocarditidis* in addition to two isolates (CCUG 18396, CCUG 18397) with identical sequences showed only minor differences from isolate CCUG 18732 and grouped together in all phylogenetic trees (Fig. 1) (isolates with identical sequences are listed in Supplementary Table S1). Isolate FD200 was only related to the type strain of *Av. endocarditidis* in the *rpoB*, *recN* and *infB* genes (Fig. 1a–c), while isolate CCUG 18551 diverged from the type strain only in *rpoB*. With respect to *rpoB*, isolates CCUG 18732, CCUG 18396, CCUG 18397, CCUG 18551, FD200, CCUG 1208, RB1857, RB1554, RB1740 and

CCUG 18395 also were related to the type strain, and *recN* related the type strain to isolates B69/96/3 and B70/96/1 (Fig. 1a–e) (isolates with identical sequences are listed in Supplementary Table S1).

With the exception of *sodA*, the type strain of *Av. paragallinarum* and isolates SP2009-1302 and 55000 always demonstrated bootstrap values above 50%. For *sodA*, the type strain was excluded from the ‘paragallinarum’ group, with 55000 and SP2009-508 as remnants (Fig. 1e). For the *recN* gene, the relationship within the *Av. paragallinarum* group (the type strain, SP2009-1302 and 55000) was more remarkable than for the other genes (Fig. 1a–e). The relationship between *Avibacterium* species A and the type strain of *Av. avium* has already been mentioned for *rpoB* and *sodA*. The reference strain of *Avibacterium* species A was related to isolates P. sp. pp. 6, PG145 and PG178 in the *recN* phylogeny. The relationship to the two latter isolates was also found for *rpoB*, whereas *pgi* and *sodA* phylogenies showed a relationship to isolate 84611 (Fig. 1a–e).

Fig. 1(c) *infB* (d) *pgi*

Consensus comparison between phylogenies obtained from the five genes showed high divergence between the topologies. Only three groups defined by bootstrap values higher than 50 % were found in the majority of trees (Table 1). The type strain of *Av. paragallinarum* and isolates SP2009-1302 and 55000 formed one group (I). The type strain of *Av. volantium* and isolate 47483/5 formed another (II), while isolates G915/84, CCUG 18403 and CCUG 18406 made up the third group (III), as already mentioned above. When groups also included sequences without perfect congruence in all five phylogenetic trees, four more groups (IV–VII) were recognized (Table 1), and they have been mentioned under the phylogenies above (Fig. 1a–e).

Consensus comparison of the five genes from type strains and the reference strain of *Avibacterium* sp. A alone showed that topologies for *Av. volantium* and *Av. paragallinarum* were supported by all five genes. Groups with *Av. endocarditidis* and *Av. avium* and *Av. gallinarum* and *Avibacterium* sp. A, respectively, were supported by two genes only, and the group with *Av. endocarditidis*, *Av. avium*,

Av. gallinarum and *Avibacterium* sp. A by three out of five genes (data not shown).

Based on the phylogenetic analysis of the five genes, including bootstrap support as well as majority rule consensus comparison (Table 1), 12 isolates were included in groups that included the type or reference strains of the five species *Av. gallinarum*, *Av. endocarditidis*, *Av. paragallinarum*, *Av. volantium*, *Av. avium* and *Avibacterium* species A, whereas six strains could not be assigned groups that included type strains of species or the reference strain of *Avibacterium* sp. A (Table 1). For four out of the six groups, the phenotype was constant within the group. Group IV included isolate FD200 classified as *Av. gallinarum* by phenotype, whereas the other members of the group belonged to *Av. endocarditidis*. Group VI included two members of *Av. gallinarum*, the type strain of *Av. avium* and the reference strain of *Avibacterium* sp. A. The rest of the isolates (31) could not be allocated to phylogenetic groups defined based on consensus comparison of the five genes investigated.

Fig. 1. (a–e) Phylogenies based on partially sequenced genes of *recN* (a), *rpoB* (b), *infB* (c), *pgi* (d) and *sodA* (e). For *recN*, ellipses represent groups with members having more than 0.86 genome similarity calculated according to the text. Isolates shown represent the sequence types listed in Supplementary Table S1. Type strains are represented by their respective species names.

DNA similarities compared between single genes

rpoB similarities varied from 90.9% between type strains of *Av. avium* and *Av. paragallinarum* to 98.8% between the type strains of *Av. endocarditidis* and *Av. gallinarum*. Excluding *Av. paragallinarum*, the lowest similarity between type strains was 96.7% between *Av. avium* and *Av. volantium*. When the unclassified isolates were included in the comparison, the lowest similarity was 91.0% (Supplementary Table S3) observed between the type strain of *Av. paragallinarum* and the type strain of *Av. avium*. For *infB*, similarities between type strains of the *Avibacterium* species varied from 88.9% (*Av. paragallinarum* and CCUG 18782) to 98.1% (CCUG 18782 and *Av. gallinarum*)

(Supplementary Table S3). The lowest similarity within the group without *Av. paragallinarum* and related isolates was 94.9% (B69/96/3 and 1248). With respect to the *Av. paragallinarum* group, the lowest similarity, 88.3% (Supplementary Table S3), was found between the type strains of *Av. paragallinarum* and *Av. volantium*. For the type strains of *Avibacterium* species, *pgi* gene sequence similarities varied from 87.4% (*Av. paragallinarum*, *Av. gallinarum*) to 96.8% (*Av. avium*, *Av. endocarditidis*). When all isolates were compared, the lowest similarity was 86.7% (Supplementary Table S3), observed between isolate 55000 and CCUG 18401. For the type strains, *sodA* similarities ranged from 90.2% (*Av. avium*, *Av. endocarditidis*) to 98.6% (*Av. paragallinarum*, *Av. volantium*). When all isolates were compared, the lowest similarity was 86.2% (Supplementary Table S3), observed between isolates 55000 and FD200.

For *recN*, nearly full-length sequence was generated from type strains in addition to 10 more isolates which were selected to represent eight *recN* monophyletic groups or singletons in Fig. 1(a). This was done to estimate the whole genomic similarity according to Kuhnert & Korczak (2006). The phylogeny obtained by comparing nearly full-length sequences of the *recN* gene is not shown, but it was congruent to the one obtained by including only partial sequences in the comparison (Fig. 1a). Nearly full-length *recN* DNA sequence similarities among type strains of *Avibacterium* species and strain CCUG 18782 (*Avibacterium* sp. A) ranged from 75.6% between *Av. paragallinarum* and *Av. gallinarum* to 96.1% between *Av. avium* and *Av. endocarditidis* (Supplementary Table S4). All type and reference strains of taxa investigated, except *Av. paragallinarum*, demonstrated very high similarities, from 94.2% between CCUG 18782 (*Avibacterium* sp. A) and the type strain of *Av. gallinarum* to 96.1% between type strains of *Av. avium* and *Av. endocarditidis*. The isolates selected to represent *recN* monophyletic groups or singletons in Fig. 1(a) were all related at the same range of 75.4–100% (Supplementary Table S3) including the type strains just mentioned. With the exception of *Av. paragallinarum* (40.1–41.0%), genome similarities between type strains varied between 82.0 and 84.7%. Including 10 additional isolates increased it to 80.2–91.0% (Supplementary Table S5).

DNA similarity based on whole genomic sequences

Calculation of ANI between the whole genomic sequences gave a value of 95% between the type strains of *Av. gallinarum* and *Av. endocarditidis*, and 83 and 84% between *Av. paragallinarum* and *Av. gallinarum* and *Av. endocarditidis*, respectively. The same result was obtained when both strains were tested either as ‘query’ or ‘subject’ in the BLAST calculation.

DNA–DNA renaturation (DDN) determined in the current investigation has been included in Supplementary Table S5. A high mean DDN of 94% was found between type strains

Table 1. Consensus comparisons of phylogenies obtained with five genes of *Avibacterium* members analysed

Strain numbers refer to Supplementary Table S1. Groups are defined by congruence in three or more of the five genes according to a majority rule (marked by an asterisk) and/or monophyly in three or more of the five genes supported by more than 50 % bootstrap values.

Group designation	Strain	Epidemiological unit	Host, organ, lesion	Country of origin	Sequence type	Consensus group
<i>Av. endocarditidis</i>	20186 ^T				1	IV
<i>Av. endocarditidis</i>	CCUG 18732	1	Chicken, heart	Australia	35	IV
<i>Av. endocarditidis</i>	CCUG 18396	2	Chicken, salpingitis, peritonitis, septicaemia	Denmark, 1979	1	IV
<i>Av. endocarditidis</i>	CCUG 18397	3	Chicken, salpingitis, peritonitis, septicaemia	Denmark, 1979	1	IV
<i>Av. gallinarum</i>	FD200	4	Avian, eye	Australia, 1980	2	IV
<i>Av. endocarditidis</i>	CCUG 18551	5	Chicken, septicaemia	Denmark, 1986	3	IV
<i>Av. paragallinarum</i>	NCTC 11296 ^T				31	I*
<i>Av. paragallinarum</i>	SP2009-1302	6	Chicken, coryza-like symptoms	The Netherlands	4	I*
<i>Av. paragallinarum</i>	55000	7	Pheasant, sinusitis	Denmark	5	I*
<i>Av. gallinarum</i>	RB410*	8	Chicken	South Africa	6	V
<i>Av. gallinarum</i>	CCUG 1208	9	Chicken, sinus	USA, 1971	7	V
<i>Av. gallinarum</i>	NCTC 11188 ^T				30	V
<i>Av. volantium-like</i>	RB1857	20	Chicken	South Africa	14	VII
<i>Av. volantium-like</i>	RB1554	18	Chicken	South Africa	14	VII
<i>Av. volantium-like</i>	RB1740	19	Chicken	South Africa	15	VII
<i>Av. gallinarum</i>	CCUG 18403	25	Chicken, purulent salpingitis	Denmark, 1980	19	III*
<i>Av. gallinarum</i>	G915/84	26	Chicken, sinusitis	Denmark	20	III*
<i>Av. gallinarum</i>	CCUG 18406	31	Chicken, septicaemia	Denmark, 1980	24	III*
<i>Av. gallinarum</i>	PG178	28	Chicken, wattle abscess	Zimbabwe	22	VI
<i>Av. gallinarum</i>	PG145	29	Chicken, pericarditis	Zimbabwe	22	VI
<i>Av. avium</i>	CCUG 12833 ^T				32	VI
<i>Avibacterium</i> sp. A	CCUG 18782				34	VI
Taxon 49†	47483/5	33	Pheasant, sinusitis	Denmark	26	II*
<i>Av. volantium</i>	CCUG 3713 ^T (=NCTC 3438 ^T)				33	II*

of *Av. gallinarum* and *Av. volantium*, whereas mean values of 67–75% were found between the other type stains investigated.

Analysis of clonality and HGT

Analysis for linkage disequilibrium of all sequences showed a significant divergence from equilibrium [standardized index of association (I_A)=0.26] of the alleles defined based on the sequence comparison of the five housekeeping genes analysed, meaning that the alleles are in linkage disequilibrium, as expected for a clonal population structure.

PLATO analysis with a window of between 75 and 200 nt showed the region between nucleotides 751 and 1491 to be significantly recombined, when the sequences of the genes were concatenated in the order *rpoB* (370 nt), *pgi* (300 nt), *recN* (352 nt), *sodA* (362 nt) and *infB* (456 nt). This region (nucleotides 751–1491) corresponds to most of the *recN* gene, the whole *sodA* gene and the 5' end of *infB*, confirming

recombination of the *sodA* gene. Simplot showed a dramatic difference between the type strain of *Av. paragallinarum* and isolate SP2009-1302 compared with the other sequences, especially in the region corresponding to *recN* (Supplementary Fig. S1). For *recN*, the result then corresponded with those from PLATO, even though the methods are based on quite different principles (similarity, SimPlot; phylogeny, PLATO).

The minimum-spanning tree showed that all isolates analysed shared at least one allele (identical sequence) (Fig. 2). The diagram shows that the sequence type that includes the type strain of *Av. endocarditis* is located at a central position with relationships to many other sequence types.

PCR-based identification

The PCR test of Chen *et al.* (1996) showed positive reactions of the type strain of *Av. paragallinarum* and

Fig. 2. Minimum-spanning tree. Numbers refer to sequence types according to Supplementary Table S1. The length of branches is proportional to the number of different alleles between sequence types. A line connecting two sequence types indicates that at least one allele is common between two sequence types.

isolates SP2008-508 and SP2008-1302, and negative reactions of 11 other isolates tested (Supplementary Table S1).

DISCUSSION

Phylogenetic comparison of 16S rRNA gene sequences demonstrated monophyly at genus and in most cases species level of prokaryotes (Ludwig & Klenk, 2001), including members of the *Pasteurellaceae* (Dewhirst *et al.*, 1993; Christensen *et al.*, 2007). Unfortunately, exceptions exist for which monophyly at species level cannot be documented based on 16S rRNA sequence comparisons, either because species are polyphyletic from the start (e.g. [*Pasteurella*] *pneumotropica*; Hayashimoto *et al.*, 2005) or because the resolution of the 16S rRNA gene sequence is insufficient to show such a relationship (e.g. *Actinobacillus equuli* and *Actinobacillus suis*; Christensen *et al.*, 2002). Phylogenetic analysis of 16S rRNA gene sequences detected polyphyly for *Av. gallinarum* (Blackall *et al.*, 2005), and difficulties were also obtained in resolving other members of the genus *Avibacterium* (Christensen *et al.*, 2009). The combination of a low degree of divergence and multiple, slightly different operons of the 16S rRNA gene might explain why species of *Avibacterium* are not monophyletic. This has previously been observed for other members of the *Pasteurellaceae* (Angen *et al.*, 2007; Nørskov-Lauritsen, 2011). For the same reason, analysis of the *recA* gene was excluded for all isolates, since multiple, different copies of the gene seemed to be present in some *Avibacterium* members.

For bacterial species, gene phylogenies might be incongruent for isolates belonging to the same species in cases of a panmictic population structure, but they should be congruent among different species (Dykhuizen & Green, 1991). The close phylogenetic relationship and high similarity between all isolates included, except for the group with *Av. paragallinarum*, could support the hypothesis that some members of *Avibacterium* are incipient species. An incipient species has not fully diverged to become a new species, and still has some traits from another species (Retchless & Lawrence, 2007). However, incipient species can be apparent as a result of HGT. This would be recognized as unresolved and reticulated network structures (Morrison, 2010). Network analysis was presented in Bisgaard *et al.* (2011), and showed unresolved networks except for *Av. paragallinarum*, indicating the HGT that may occur between incipient species. The network analysis also confirmed the high *recN* gene divergence between the *Av. paragallinarum* group (the type strain, SP2009-1302 and isolate 55000) and groups of other isolates. This divergence was also recognized with *rpoB*. A high *sodA* similarity of 98.6% was found between *Av. volantium* and *Av. paragallinarum*, and this is indicative of HGT of *sodA* into the type strain of *Av. Paragallinarum*, as indicated from the phylogenetic analysis. Simplot in the current study showed a dramatic difference between the type strain of *Av. paragallinarum* and isolate SP2009-1302 compared with

the other sequences, especially in the region corresponding to *recN*, indicating a source for this gene outside *Avibacterium*. Incipient species are expected to be mosaics, where only genes selected for are able to recombine (Via, 2009). Therefore, until the functional roles of all genes in the genomes of species of *Avibacterium* have been fully investigated, conclusions about the incipient nature of these species cannot be drawn.

A high degree of HGT was anticipated from the incongruence between phylogenetic trees including a comparison of all five gene sequences. The 24 isolates included with the consensus groups seem to be relatively more conserved in their genome organization than the other isolates investigated (see Table 1). In addition to groups that included type strains of the five species and the reference strain of *Avibacterium* sp. A, we also identified two other groups (III and VII). This could indicate new species; however, based on the high level of DNA similarity observed between the groups, this seems unlikely, as discussed in the following paragraphs.

Even comparison only of type strains of *Avibacterium* resulted in a majority rule consensus tree for only three out of the five genes. For comparison, similar consensus comparisons of the same five genes were always obtained from type strains of species of *Mannheimia*. Here, *Mannheimia haemolytica* and *Mannheimia glucosida* formed a monophyletic group, and *Mannheimia varigena* and *Mannheimia granulomatis* another, with *Mannheimia ruminalis* situated between the groups for all five genes (data not shown).

The *recN* similarities converted to genome similarity demonstrated 80–95% genome similarity without the inclusion of *Av. paragallinarum*, and 40–95% including *Av. paragallinarum* in the comparison (Supplementary Table S5). We compared similar genome similarities for the type strains of *Mannheimia* species and found the range to vary from 40.6 to 52.0% genome similarity for four species. For *M. haemolytica* and *M. glucosida*, 91.9% similarity was found, and these species are well known to be closely related (Angen *et al.*, 1999) (see Supplementary Table S3). The *recN* phylogenetic comparison therefore showed a very narrow species relationship between type strains of *Av. volantium*, *Av. avium*, *Av. endocarditidis* and *Avibacterium* species A. Using the interpretation of Kuhnert & Korczak (2006), members of a species demonstrate genome similarities higher than 90%, and interval of similarity values between 0.85 and 0.9 have been taken as intermediate, and may indicate different species or subspecies. Using this interpretation, some species of *Avibacterium* can still be separated. Four groups of isolates were delineated at a 0.86 genome similarity (see Fig. 1a). The largest group included type strains of *Av. volantium*, *Av. avium*, *Av. endocarditidis* and 33 isolates, including the reference strain of *Avibacterium* species A. This group included members of five of the consensus groups identified (see Table 1). The type strain of *Av. gallinarum* and five additional isolates

formed a closely related group that partly corresponded to the consensus group V (Table 1). The lower genome similarity of 0.40–0.42 for *Av. paragallinarum* indicates a distinct relationship even at the genus level (group I, Table 1). Transfer of *recN* to this species from a source outside of *Avibacterium* might explain this result. For *rpoB*, the similarity between species of *Avibacterium* was similar to those for *Mannheimia* and some members of the *Enterobacteriaceae*, for which 89.4–96.6 % similarity between species has been observed (Giammanco *et al.*, 2011). Also, within *Streptococcus* similarities in this high range have been found (76.1–97.6 %) between species (Glazunova *et al.*, 2009). A general cut-off value for *rpoB* of 97.7 % between species was given by Adékambi *et al.* (2009) for the whole gene and 96–97 % for a partial sequence as used in the current investigation. With this criterion, only *Av. paragallinarum* represents a true species within *Avibacterium* if comparison is based on type and reference strains only. For the *pgi*, *sodA* and *infB* genes, sequence similarities of *Avibacterium* were higher than those for *Mannheimia* (see Supplementary Table S3).

To investigate the narrow relationships between some species of *Avibacterium* we performed pair-wise comparisons of the whole genomic sequences of type strains of *Av. gallinarum*, *Av. paragallinarum* and *Av. endocarditidis*, resulting in ANI values of 83, 84 and 95 %. These values were converted to 35, 37 and 70 % DDN, respectively, by use of the formula in Goris *et al.* (2007) (Supplementary Table S5) (see Discussion for comparison of these data with DDN). The 35 % determined by ANI is in excellent correspondence to the 34 % DDN determined by Mutters *et al.* (1985). Earlier, 60 % DDN was found between *Av. avium* and *Av. volantium* type strains (Mutters *et al.*, 1985). According to the current practice of separating species which are less than 70 % DDN related, these taxa were therefore separated as distinct species. Unfortunately, the results reported by Mutters *et al.* (1985) could not be repeated with the same method of DNA–DNA hybridization in the current study. While Mutters *et al.* (1985) reported a value of 60 % DDN for *Av. avium* and *Av. volantium* (sufficient to justify a separate genomospecies), the current study found higher values of 66 % and 70 %, which do not clearly support separate genomospecies. The current study also demonstrated severe problems in separating *Av. avium* and *Avibacterium* species A at the genomospecies level.

The present investigation underlines the problem associated with circumscription of species based upon a single or a few isolates (Christensen *et al.*, 2001). When a diverse strain collection is investigated subsequently, it becomes very difficult to classify isolates with proper species, not to mention meeting the criteria suggested for identification. Unfortunately, the conclusion is that with the exception of the lower genome similarity of 0.40–0.42 for *Av. paragallinarum*, other species of *Avibacterium* might be difficult to delineate in a laboratory performing routine identification. We found 20 % (10 out of 49) of the field isolates characterized to vary in one or more characteristics

compared with the original description of the species of *Avibacterium*. A similar observation of deviating isolates was made by Blackall (1988). Even the identification of species of *Avibacterium* by extensive DNA sequencing seems problematic, since *Av. avium* and *Av. volantium* or *Av. avium* and *Avibacterium* sp. A cannot be separated at the genomospecies level. The most likely explanation is that *Av. volantium*, *Av. avium* and *Avibacterium* sp. A were misclassified originally. Eventual reclassification will have to await further phenotypic and genotypic characterization of members of *Avibacterium*, especially within the *Av. paragallinarum* lineage. Identification of *Av. paragallinarum* by PCR was positive for the type strain and isolates SP2008-1302 and SP2008-508 isolated from chicken. A total of 11 other isolates were negative including isolate 55000 isolated from pheasant. This confirms that the PCR gave a positive result in the detection of isolates of *Avibacterium* from chicken with coryzae or coryzae-like symptoms; however, the results are not in correspondence with the phylogeny derived from the five housekeeping genes investigated in the current study.

In conclusion, the present investigation indicates that *Avibacterium* should be reclassified, leaving only two or three species in the genus. Until the taxonomic revision is completed, we recommend that isolates that do not fit with named species by genotypic and/or phenotypic methods are designated *Avibacterium* sp.

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