Multilocus sequence phylogenetic analysis of *Avibacterium*

M. Bisgaard,¹ N. Nørskov-Lauritsen,² S. J. de Wit,³ C. Hess⁴ and H. Christensen¹

¹Department of Veterinary Disease Biology, Faculty of Life Sciences, University of Copenhagen, 4 Stigbøjlen, DK-1870 Frederiksberg C, Denmark
²Department of Clinical Microbiology, Aarhus University Hospital Skejby, Denmark
³GD, Postbus 9, 7400 AA Deventer, The Netherlands
⁴Clinic for Avian, Reptile and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University for Veterinary Medicine Vienna (Vetmeduni Vienna), Veterinaerplatz 1, 1210 Vienna, Austria

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*, *gnd*, *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.

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 endocarditidis*, 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
members of the Pasturellaceae (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 Pasturellaceae 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 Pasturellaceae (Norskov-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 (HTG) 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 respects are related to validly published species, and to investigate whether indications of HTG 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′-ATGCTTANYCAWCTYACKATYAAATMTTTCG-3′) and recN_first-R (5′-CCRCTAATYCCMACATCNACYCTCATC-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′-GTAAGTATTGATGCGGCTGGT-3′) and recN-2 (5′-CAGAGCCGCTAATATTAC-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 et al. (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 rpoBfPm (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 Pgi838F and Pgi1331R 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 Pasturellaceae, 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 Norskov-Lauritsen et al. (2005). In addition to primers included in that publication, the primers recA54f5 (5′-GAAAARCAATTTGGKAAGGYG-3′) and recA617rv (5′-TIACTACACTHACVCCTAAT-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 CONSENS 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 EMBOS (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 Hülse 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, EMBOS (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/avibacterium/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 epidemi-
ologically related isolates, identity was initially achieved with
the rpoB sequences and only one isolate (1248) was selected
for further characterisation (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 recN 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 recN 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 recN 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
two 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. 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.
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).
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. endocarditis and Av. avium and Av. gallinarum and Avibacterium sp. A, respectively, were supported by two genes only, and the group with Av. endocarditis, 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. endocarditis, 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. endocarditis. 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.
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 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 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.

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

Multilocus sequence analysis of *Avibacterium*
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 (IA)=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 *recN* 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. endocarditis, 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 Mutter et al. (1985). Earlier, 60% DDN was found between Av. avium and Av. volantium type strains (Mutter 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 Mutter et al. (1985) could not be repeated with the same method of DNA–DNA hybridization in the current study. While Mutter 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.

ACKNOWLEDGEMENTS

Excellent technical assistance was contributed by the technician Katrine Madsen. We owe a great debt of gratitude to scientists who have contributed isolates to this investigation.

REFERENCES


Felsenstein, J. (1995). PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author, Department of Genome Sciences, University of Washington, Seattle, USA.


Edited by: S. D. Bentley