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Analysis of the 16S-23S rRNA intergenic spacer region of *Mycoplasma synoviae* field strains

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## Abstract

*Mycoplasma synoviae* has been associated with economic loss in chicken and turkey industries. The molecular characterization of *M. synoviae* at strain level allows the analysis of relationships between strains which may be valuable in epidemiological investigations. In this study, the intergenic spacer region (ISR) between the 16S and 23S rRNA genes was examined to see if useful information about strains could be derived. *M. synoviae* has two copies of this region, which may not be exactly the same (intercistronic heterogeneity). Sequencing of the ISRs of 21 *M. synoviae* isolates and the type strain revealed that 19 of them had such heterogeneity so DNA cloning was performed where necessary. All sequences were analysed and aligned; the percentage similarity of the DNA was calculated and a dendrogram was constructed. The length of the ISRs varied between 305 and 309 bp. Apart from having extra A/Ts in poly A or poly T regions and the presence of a few polymorphisms, the sequences of the *M. synoviae* strains were similar. Based on phylogenetic analysis the strains were assigned to 10 groups, taking into account that within each group the DNA similarity was 100%, while the lowest similarity between groups was 95.8%. The results were compared to those obtained with the *vlhA* gene, resulting in very similar *M. synoviae* groups. Although the ISR could be a good target for strain typing, as has been shown by others for *Mycoplasma gallisepticum*, the method may be too cumbersome for routine use with *M. synoviae* because of complications with intercistronic heterogeneity. However, if the ISR sequence information were to be combined with other mutation detection techniques it could increase the discriminatory power.

## Introduction

*Mycoplasma synoviae* has been found in a wide range of birds and appears to be particularly prevalent in chickens and turkeys (Kempf, 1998, Hagan *et al.*, 2004). It can cause infectious synovitis and subclinical respiratory infections and also overt respiratory disease under some circumstances (Bradbury & Morrow, 2008). The epidemiology of *M. synoviae* is complex since it can be transmitted either vertically through the egg, or horizontally to other birds by direct contact between clinically affected birds or sub-clinical carriers and susceptible birds. Indirect transmission may also play a role in spread of the organism (Marois *et al.*, 2000) and, even though the mycoplasmas do not apparently survive for long periods in the environment, *M. synoviae* may persist on feathers for up to 2 or 3 days at room temperature (Christensen *et al.*, 1994). Indeed, an experimental study has shown that inanimate objects contaminated with *M. synoviae* (dust, feathers and food) can infect chicks (Marois *et al.*, 2005).

There is no obvious phenotypic diversity among *M. synoviae* strains but molecular characterization at strain level may assist in epidemiological studies to determine the source of infections and the relationships among strains isolated from neighbouring or related flocks. Heterogeneity among *M. synoviae* strains has been studied by ribotyping (Yogev *et al.*, 1988), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell proteins (Ley & Avakian, 1992), restriction endonuclease analysis (REA) of DNA (Morrow *et al.*, 1990; Ley & Avakian, 1992), random amplified polymorphic DNA (RAPD) (Fan *et al.*, 1995; Rawadi, 1998; Marois *et al.*, 2001; Feberwee *et al.*, 2005), pulsed-field gel electrophoresis (PFGE) (Marois *et al.*, 2001), and amplified fragment length polymorphism (AFLP) (Feberwee *et al.*, 2005; Hong *et al.*, 2005). More recently, it was shown that DNA

sequence analysis targeting the haemagglutinin-encoding *vhhA* gene is a useful tool for detection and initial typing of *M. synoviae* strains (Benčina *et al.*, 2001; Hong *et al.*, 2004; Jeffery *et al.*, 2007, Hammond *et al.*, 2009).

Another DNA sequencing target is the intergenic spacer region of the 16S-23S rRNA genes (ISR). The ISR separates each rRNA gene, normally organized in operons and arranged in the order 5' 16S-23S-5S 3'. Knowledge about the organization of the mycoplasma rRNA operon and the number of such operons is still somewhat limited but the most common number is one or two (Razin *et al.*, 1998). *M. synoviae* has two sets of 16S and 23S rRNA genes and three sets of 5S rRNA genes (Vasconcelos *et al.*, 2005; De Oliveira Santos *et al.*, 2007). The 16S and 23S rRNA genes are organized in two operons in the same orientation (Allen *et al.*, 2005) while the 5S rRNA genes are apparently located at a greater distance than usual from the 16S-23S cluster (De Oliveira Santos *et al.*, 2007). In previous work, the ISR from the type strains of all the avian *Mycoplasma* species was studied (Harasawa *et al.*, 2004; Ramírez *et al.*, 2008) and great variations in size and composition were found. The only conserved region between all the avian mycoplasmas (Ramírez *et al.*, 2008) was a sequence described by Harasawa *et al.*, (1992) as 'box A'.

Gürtler and Stanisich (1996) suggested that considerable variation of the ISR can occur not only between species but also between strains within a species. However there can also be variation between alleles of the rRNA operon within a single strain. Sequences of this region have been useful for analysis of strains of various bacterial species, such as *Bifidobacterium* spp. (Leblond-Bourget *et al.*, 1996), *Streptococcus* spp. (Chanter *et al.*, 1997), *Bacillus subtilis* (Shaver *et al.*, 2001), phytoplasmas (Smart *et al.*, 1996), *Ureaplasma urealyticum* and *Ureaplasma parvum* (Harasawa & Kanamoto, 1999; Robertson *et al.*, 2002), and *Mycoplasma gallisepticum* (Raviv *et al.*, 2007; Yavari *et al.*, 2007).

As there appears to be no information available about the ISR sequences of *M. synoviae* field strains the aim of the present study was to determine the detail of the intra-specific polymorphism of the *M. synoviae* ISR, to evaluate the discriminatory power and to analyse the potential usefulness of this region for identifying *M. synoviae* strains in epidemiological studies. The results were then compared to those obtained by analysing the *vlhA* gene, using data mainly from an earlier study (Hammond *et al.*, 2009).

## Materials and methods

**Organisms and culture conditions.** *M. synoviae* type strain WVU1853<sup>T</sup> and 21 *M. synoviae* field isolates from different hosts, different countries and at different periods were used in an attempt to cover a significant section of the genetic diversity of this bacterial species (Table 1). The mycoplasmas were cultivated in mycoplasma broth and agar (Bradbury, 1977) in a CO<sub>2</sub> rich atmosphere. They were filter-cloned (Tully, 1983) to ensure purity, and the identity and purity were confirmed by the indirect fluorescent antibody (IFA) test (Rosendal and Black, 1972).

**DNA extraction.** DNA was extracted using Chelex resin and heating as has been previously described (Harasawa *et al.*, 2004).

**PCR primers and amplification of the 16S-23S ISR.** DNA amplifications were performed with a forward primer (5'-CGT TCT CGG GTC TTG TAC AC-3') and a reverse primer (5'-CGC AGG TTT GCA CGT CCT TCA TCG-3') in a DNA thermal cycler (Perkin-Elmer), following the protocol used by Ramírez *et al.* (2008). These primers were designed to amplify the ISR of all the avian mycoplasmas.

**PCR primers and amplification of the *vlhA* gene.** In order to compare the phylogenetic tree obtained from the ISR sequences with that derived from the *vlhA* gene sequences of the same isolates, PCR amplification using *vlhAF* and *vlhAR2* primers was performed with five of the *M. synoviae* isolates as previously described (Hammond *et al.*, 2009). These are indicated on Table 1. The other *vlhA* gene sequences had been already published by Hammond *et al.* (2009).

**Cloning PCR products and sequencing of the ISR.** Where DNA cloning was needed, conventional methods for the ligation, transformation and recombination were used (Ramírez *et al.*, 2008). The plasmids were purified with DNA Spin MiniPrep Kit (Qiagen) according to the manufacturer's instructions. The size of the inserts was confirmed by 1.5% agarose gel electrophoresis after *EcoR1* enzyme (Gibco BRL) digestion. For each strain five clones were selected for sequencing. Sequencing was conducted by Imperial College, London (UK) and for every PCR product both strands of DNA were sequenced at least twice.

**Data analysis.** The resulting chromatograms were examined in Chromas (version 1.45; School of Health Science, Griffith University, Australia). The forward and reverse complemented sequences were compared to produce a consensus sequence using Generunner (version 3.05; Hastings Software, Inc.). Dendrograms were constructed from the ISR and *vlhA* alignments by the neighbour-joining method and 1000 bootstrap replicate analysis using the Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0 (Tamura *et al.*, 2007).

For checking the intra-species variability with percent similarity of the ISR sequences of all *M. synoviae* strains was determined using the program Bioedit

version 7.0.0 (Hall, 1999). Strains with identical sequences were assigned to the same alphabetical group.

The numerical index of discrimination ( $D$ ) used was described by Hunter & Gaston (1988).  $D > 0.95$  is considered a good discrimination index.

**Nucleotide sequence accession numbers.** The *M. synoviae* sequences determined here were assigned in the Genbank with accession numbers FM21339 to FM213419 for the ISR and FN666084 to FN666088 for the new *vlhA* sequences.

## Results

ISR PCR products were successfully obtained from all the *M. synoviae* strains, although there was a three nucleotide mismatch in the middle of the reverse primer. The amplified fragments (around 540 bp), which yielded a single product in each case, corresponded in size to the length of the ISR plus 163 bp from the 3' end of the 16S rRNA gene and 71 bp from the 5' end of the 23S rRNA gene (data not shown). The length of the ISRs, the polymorphisms, the deletion/insertions and the Genbank/EMBL accession numbers for the ISR sequences of the 22 *M. synoviae* strains are listed in Table 1 and details of the polymorphisms are shown in Table 2. Complete sequence information is in Supplementary Figure 1. The length of the ISRs varied between 305 and 309 bp. The number of polymorphisms found within a single *M. synoviae* strain varied between 0 and 5. The number of nucleotide differences between different *M. synoviae* strains varied from 0 to 11.

In the alignment (Figure 1) it can be seen that apart from having extra A/Ts in poly A or poly T regions and the presence of a few polymorphisms, the sequences of



the *M. synoviae* strains are exactly the same. The strains could be allocated to 10 groups (A to J) based on 100% DNA similarity (Table 1), members of each group having exactly the same polymorphisms and insertion/deletion patterns (Table 2). The percent similarity between the groups is shown in Table 3, with the lowest similarity being 95.8% and the highest 99.3%.

The phylogenetic analysis is shown as a dendrogram (Figure 2). This information complements the sequence information and shows the relationship between each group. Seven of the 10 groups have only one member, two have two and group E contains 11 strains. The numerical index of discrimination for these ISR sequences was 0.75.

Amplification of the *vlhA* gene of the strains B22/91 B98/95, B19/02, B76/02 and Sp267 produced a single PCR product per strain. The length of the sequence varied between the strains from 354 bp (B98/95 and B76/02) to 315 bp of the strain Sp267, being the strains B22/91 and B19/02, 327 bp. The dendrogram based on the *M. synoviae vlhA* sequences (Figure 3) showed very similar groups to that shown in the ISR in Figure 2 except that using the *vlhA* sequences strain B133/99/5 split from group E into a new group (K). This was due to the deletion of 39 nucleotides. The other difference was that strains B13/96/P1 and B13/96/P2 were in the same group whereas they were separated into two groups (F and G) when the ISR sequences were analysed because of two polymorphisms found in B13/96/P1 ISR sequence (Table 2).

## Discussion

The ISR primers used in our study were 'universal' in that they were designed to amplify the ISR of all avian *Mycoplasma* species (Ramírez *et al.* 2008). The

reverse primer has also been paired successfully with a specific forward *M. synoviae* primer based in the ISR for a diagnostic PCR (Ramírez *et al.*, 2006). However, to have a perfect match with the published WVU1853 23S rRNA sequence, the primer sequence should be 5'-CGC AGG TAA TCA CGT CCT TCA TCG-3' instead of 5'-CGC AGG TTT GCA CGT CCT TCA TCG-3'. All our *M. synoviae* isolates and the type strain were successfully amplified despite the three mismatches present in the reverse primer.

The ISR is assumed to have more variability than the highly conserved 16S rRNA gene (Gürtler, 1999) and variation within the ISR of *M. synoviae* strains was suggested by Lauerman *et al.* (1995) when they observed variable lengths of PCR amplicons among *M. synoviae* strains when universal primers for amplification of the 16S/23S rRNA ISR were used. There was also variation in their banding patterns after restriction fragment length polymorphism analysis of the PCR products. Therefore we expected some degree of variation in the ISR of our *M. synoviae* strains. However in our study, all strains yielded only one PCR product of the same size, approximately 540 bp, meaning that there were no great differences in sequence length in our strains.

After sequencing, some variability in length and in sequence was found between the *M. synoviae* strains (intra-species) (Figure 1) and also between the two operons (intercistronic heterogeneity) (Table 1). The length variability was due to extra As or Ts in poly A or poly T regions, respectively.

In bacteria, where there is more than one operon these are often not identical, particularly in relation to the ISR (Condon *et al.*, 1995). In our study, we assumed that since universal primers were used, all operons were targeted; however, the locus of the ISRs examined in this study could not be identified. Only

three of the *M. synoviae* strains presented intercistronic homogeneity (strains B38/96/1764, B19/02 and Sp267).

Polymorphisms and/or sequence length variations in the ISR were found in all the other strains. In this situation, the sequences were not always possible to determine and cloning was needed to obtain them. Volokhov *et al.* (2006) suggested two possible explanations: the presence of at least two ISR alleles in the chromosome or the existence of a subtype strain mixture. As colony cloning was done in this study, the first suggestion is the most plausible, since recent studies confirmed that *M. synoviae* has two rRNA operons (Allen *et al.*, 2005; Vasconcelos *et al.*, 2005; De Oliveira Santos *et al.*, 2007). *M. gallisepticum*, on the other hand, contains only a single copy of the ISR between the 16S and the 23S rRNA genes (Papazisi *et al.*, 2003). For this reason, Raviv *et al.* (2007) and Yavari *et al.* (2007) did not find any of those problems when using the ISR to type the avian pathogen *M. gallisepticum*.

The numbers of sequence differences in *M. synoviae* are larger than in other *Mollicutes* spp. examined, showing an intra-species similarity of 95.8% compared to 100% between strains of *M. iowae* (Ramírez *et al.*, 2008), *M. pulmonis* (Harasawa, 1999), *M. pneumoniae* (Dorigo Zetsma *et al.*, 2000), *Ureaplasma urealyticum* and *U. parvum* (Harasawa & Kanamoto, 1999; Robertson *et al.*, 2002). Volokhov *et al.* (2006) analysed sequences of the ISR of a few (between two and eight) strains of each of *M. hominis*, *M. arginini*, *M. bovis*, *M. genitalium*, *M. pneumoniae*, *M. hyorhinis* and *M. felis* obtained from GenBank and found 98–100% similarity within species. However our previous studies with 16 *M. meleagridis* strains showed 97.2% similarity, while far more variability (94%) was seen within 11 strains of *M. gallisepticum* (Ramírez *et al.*, 2008).

The apparent variation in the ISRs of some species and not in others might be related to the opportunities for host to host passage of the organism accelerating the rate of mutation. *M. synoviae* and *M. gallisepticum* have many host species (chicken, turkey, game and wild birds) and also infect very large populations of chickens and turkeys while, for example, mycoplasmas with human or bovine hosts will have less opportunity for host to host passage.

Although it was possible to put the 22 *M. synoviae* strains into 10 groups, 11 of them fell into one group (E) while seven strains each constituted an individual group. The index of discrimination  $D$  was poor, being 0.75. An index  $>0.90$  is considered adequate (Raviv *et al.*, 2007). However, the acceptable level of discrimination will depend on several factors including the number of strains used. Hunter & Gaston (1988) gave an example with 100 strains, while only 22 strains were tested in this study.

Another reason for the low index was that the analysis should ideally be carried out between unrelated strains. Our strains were obtained mainly from the laboratory archive from our diagnostic service and some of them could be related cases, but within the poultry industry it is very difficult to establish the direct and indirect contact between infected flocks. For the 11 strains that shared the same sequence profile (Group E), associations could be seen between some strains. For example strains B91/96/798 and B91/96/801 were isolates from the same flock, as were strains B133/99/5 and B133/99/12. Even though some strains in this group were isolated from poultry located in different countries, the opportunity for spread of *M. synoviae* strains between these countries by commercial trading cannot be discounted. Strain B13/96/P1 (Group F) and strain B13/96/P2 (Group G) were isolated at the same time from two pheasants near an *M. synoviae*-infected chicken flock and it was expected that they might also be identical but the ISR analysis places them in two

different groups. From these results it is not possible to know if the variation is due to passage within pheasants or if each was infected by a different strain.

Despite the poor value of the index of discrimination we were still able to divide 22 strains into 10 groups which suggests that there may be some value for using the ISR for epidemiological purposes as has already been shown for *M. gallisepticum* (Raviv *et al.*, 2007), as well as for other bacteria such as *Clostridium difficile* (Gürtler, 1993), *Bacillus* spp. (Houpikian & Raoult, 2001), *Streptococcus zooepidemicus* (Newton *et al.*, 2008) and *Vibrio vulnificus* (González-Escalona *et al.*, 2007).

The sequences of the *vlhA* genes of strains B22/91, B98/95, B19/02, B76/02 and Sp267 were used together with those of Hammond *et al.* (2009) to build a dendrogram, in order to compare it with the one obtained using the ISR information. The two dendrograms (Figure 2 and Figure 3) produced nearly the same groupings, surprisingly, because the ISR is a non-coding region and the *vlhA* gene codes for a haemagglutinin. Hammond *et al.* (2009) mention that strains B133/99/5 and B133/99/12 were isolates from the same farm. Their ISRs are exactly the same but the *vlhA* sequences differ in a single insertion/deletion of 39 nucleotides. Hammond *et al.* (2009) suggested that either both isolates were the same strain and one had undergone a deletion during passage on the farm, or that the isolates represented two different strains. Looking at the comparative result, we believe that the first hypothesis is more likely. The converse situation was seen with the sequences of strains B13/96/P1 and B13/96/P2, which by *vlhA* analysis appeared to be the same, but which showed two polymorphisms in the ISR in the former strain that do not exist in the latter. Both strains were isolated from pheasants from the same location.

Both methods appear to give useful and similar information for *M. synoviae* strain typing. However the advantage of using the *M. synoviae vlhA* gene method is that a specific PCR is used and there is no need to cultivate or clone the organism.

In conclusion although analysis of the *M. synoviae* ISR is a potentially useful tool for strain typing cloning of the DNA may be needed for strains in which the two copies are different in length. This may make the method too cumbersome for routine use. However, ISR typing may prove useful with other mutation detection techniques such as denaturing gradient gel electrophoresis, single-strand conformation polymorphism or high-resolution melting curve analysis.

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**Figure legends**

**Figure 1.** An alignment of *M. synoviae* 16S-23S rDNA ISR sequences according to group designation. Identities are indicated by solid box. Positions where nucleotide differences exist in one of the two sequences of an *M. synoviae* strain are denoted with the ambiguity letter code according to IUB. The sequence length variation is indicated with lower-case letters. G = Guanine; A = Adenine; T = Thymine; C = Cytosine; Y = T/C; W = A/T; M = A/C; K = T/G.

**Figure 2.** Dendrogram based on the *M. synoviae* 16S-23S rDNA ISR sequences. The neighbour-joining method with 1000-bootstrap replicates was used in MEGA 4.0. Bootstrap percentage values are located at nodes of the tree. The group letter corresponds to those described in Table 1.

**Figure 3.** Dendrogram based on the *M. synoviae* v1hA sequences. The neighbour-joining method with 1000-bootstrap replicates was used in MEGA 4.0. Bootstrap percentage values are located at nodes of the tree. To make the comparison with Figure 2 easier, the same group letters are used except for the addition of group K.

**Supplementary Figure 1.** Alignments of each group (A to J) of *M. synoviae* 16S-23S rDNA ISR sequences showing the published sequence alongside those of the two alleles after DNA cloning

**Table 1.** *Mycoplasma synoviae* strain details

<i>M. synoviae</i> strains <sup>a</sup>	Host <sup>b</sup>	Date <sup>c</sup>	Country <sup>d</sup>	ISR (n)	Polymorphisms <sup>e</sup>	Group <sup>f</sup>	Accession numbers (ISR) <sup>g</sup>	Accession numbers (VlhA) <sup>h</sup>
WVU1853 <sup>T</sup>	Ch	1957 <sup>i</sup>	USA	308	17 <sub>w</sub> , 58 <sub>KWWaaa</sub> , 282 <sub>Y</sub>	A	AJ781002	AM998371
B11/85	Tu	1985	UK	305	93 <sub>t</sub>	B	<b>FM213399</b>	FM164346
J26/85	Ch	1985	UK	305	23 <sub>M</sub> , 239 <sub>Y</sub>	C	<b>FM213400</b>	AJ580982
B7/86	Ch	1986	UK	305	93 <sub>t</sub>	B	<b>FM213401</b>	FM164347
B22/91	Ch	1991	SA	309	37 <sub>tt</sub> , 43 <sub>Y</sub> , 58 <sub>KWWaaa</sub>	D	<b>FM213402</b>	<b>FN666084</b>
B98/95	Ch	1995	NE	307	17 <sub>w</sub> , 94 <sub>t</sub> , 140 <sub>a</sub> , 284 <sub>taacaata</sub>	E	<b>FM213403</b>	<b>FN666085</b>
B13/96/P1	Ph	1996	UK	309	17 <sub>w</sub> , 58 <sub>KWWaaa</sub> , 66 <sub>Y</sub> , 95 <sub>Y</sub> , 204 <sub>t</sub>	F	<b>FM213404</b>	AJ580987
B13/96/P2	Ph	1996	UK	309	17 <sub>w</sub> , 58 <sub>KWWaaa</sub> , 204 <sub>t</sub>	G	<b>FM213405</b>	FM164343
B38/96/1764	Tu	1996	Italy	306	-	H	<b>FM213406</b>	AJ580985
B91/96/798	Tu	1996	UK	307	17 <sub>w</sub> , 94 <sub>t</sub> , 140 <sub>a</sub> , 284 <sub>taacaata</sub>	E	<b>FM213407</b>	FM164349
B91/96/801	Tu	1996	UK	307	17 <sub>w</sub> , 94 <sub>t</sub> , 140 <sub>a</sub> , 284 <sub>taacaata</sub>	E	<b>FM213408</b>	FM164350
B55/97/2a	Ch	1997	UK	307	17 <sub>w</sub> , 94 <sub>t</sub> , 140 <sub>a</sub> , 284 <sub>taacaata</sub>	E	<b>FM213409</b>	FM164353
B3/98	Ch	1998	NE	307	17 <sub>w</sub> , 94 <sub>t</sub> , 140 <sub>a</sub> , 284 <sub>taacaata</sub>	E	<b>FM213410</b>	FM164354
B133/99/5	Ch	1999	Hungary	307	17 <sub>w</sub> , 94 <sub>t</sub> , 140 <sub>a</sub> , 284 <sub>taacaata</sub>	E	<b>FM213411</b>	AJ580983
B133/99/12	Ch	1999	Hungary	307	17 <sub>w</sub> , 94 <sub>t</sub> , 140 <sub>a</sub> , 284 <sub>taacaata</sub>	E	<b>FM213412</b>	FM164358
B27/00	Ch	2000	Hungary	307	17 <sub>w</sub> , 94 <sub>t</sub> , 140 <sub>a</sub> , 284 <sub>taacaata</sub>	E	<b>FM213413</b>	AJ580988
B19/02	Ch	2002	Spain	306	-	H	<b>FM213414</b>	<b>FN666086</b>
B76/02	Ch	2002	Italy	307	17 <sub>w</sub> , 94 <sub>t</sub> , 140 <sub>a</sub> , 284 <sub>taacaata</sub>	E	<b>FM213415</b>	<b>FN666087</b>
B142/02	Ch	2002	Hungary	307	17 <sub>w</sub> , 38 <sub>t</sub> , 93 <sub>t</sub> , 141 <sub>a</sub>	I	<b>FM213416</b>	AJ580989
B 155/02	Ch	2002	UK	307	17 <sub>w</sub> , 94 <sub>t</sub> , 140 <sub>a</sub> , 284 <sub>taacaata</sub>	E	<b>FM213417</b>	FM164360
B35/03 (2)	Ch	2003	UK	307	17 <sub>w</sub> , 94 <sub>t</sub> , 140 <sub>a</sub> , 284 <sub>taacaata</sub>	E	<b>FM213418</b>	FM164365
Sp267	Sp	2001	MA	308	-	J	<b>FM213419</b>	<b>FN666088</b>



<sup>a</sup>All *M. synoviae* field strains were isolated at Liverpool University (UK), with the exception of strain *M. synoviae* sp267, isolated by K.

Ganapathy in Universiti Putra Malaysia

<sup>b</sup>Ch = chicken; Tu = turkey; Ph = pheasant and Sp = sparrow

<sup>c</sup>Year of isolation

<sup>d</sup>Country of isolation; UK = United Kingdom; SA = South Africa; MA = Malaysia; NE = Netherlands

<sup>e</sup>Nucleotide positions are according to the numbering of the *M. synoviae* type strain sequence in GeneBank (AJ781002) and the designation of the polymorphisms used the IUB letter code. A lower-case letter indicates a nucleotide present in only one of the operons

<sup>f</sup>*M. synoviae* strains are classified according to the presence of polymorphisms, insertions/deletions and the length of the sequence

<sup>g</sup>All *M. synoviae* ISR were sequenced for this study (with bold accession numbers) except the type strain which was sequenced in a previous study (Ramírez *et al.*, 2008).

<sup>h</sup>Bold accession numbers were sequenced for this study. The other sequences were done for the paper Hammond *et al.* (2009).

<sup>i</sup>Year of first publication.

**Table 2.** *Details of the polymorphisms in the two copies of the ISRs in the different groups of M. synoviae*

Group	
A	17 <sub>T</sub> , 58 <sub>TTT---</sub> , 282 <sub>C</sub> 17 <sub>A</sub> , 58 <sub>GAAAAA</sub> , 282 <sub>T</sub>
B	93. 93 <sub>T</sub>
C	23 <sub>C</sub> , 239 <sub>T</sub> 23 <sub>A</sub> , 239 <sub>C</sub>
D	37 <sub>-</sub> , 43 <sub>C</sub> , 58 <sub>TTT---</sub> 37 <sub>TT</sub> , 43 <sub>T</sub> , 58 <sub>GAAAAA</sub>
E	17 <sub>T</sub> , 94 <sub>-</sub> , 140 <sub>-</sub> , 284 <sub>-----</sub> 17 <sub>A</sub> , 94 <sub>T</sub> , 140 <sub>A</sub> , 284 <sub>TAACAATA</sub>
F	17 <sub>T</sub> , 58 <sub>TTT---</sub> , 66 <sub>C</sub> , 95 <sub>C</sub> , 204. 17 <sub>A</sub> , 58 <sub>GAAAAA</sub> , 66 <sub>T</sub> , 95 <sub>T</sub> , 204 <sub>T</sub>
G	17 <sub>T</sub> , 58 <sub>TTT---</sub> , 204. 17 <sub>A</sub> , 58 <sub>GAAAAA</sub> , 204 <sub>T</sub>
H	-
I	17 <sub>T</sub> , 38 <sub>-</sub> , 93 <sub>T</sub> , 141. 17 <sub>A</sub> , 38 <sub>T</sub> , 93 <sub>-</sub> , 141 <sub>A</sub>
J	-

**Table 3.** *ISR sequence percent homology among M. synoviae field strain groups*

<i>M. synoviae</i> groups	A	B	C	D	E	F	G	H	I	J
A	ID									
B	97.4	ID								
C	96.7	99.3	ID							
D	98.7	97.0	96.4	ID						
E	97.0	99.0	98.3	96.4	ID					
F	98.7	96.7	96.1	98.0	96.4	ID				
G	99.3	97.4	96.7	98.7	97.1	99.3	ID			
H	96.4	98.6	98.0	97.0	98.7	95.8	96.4	ID		
I	97.0	99.0	98.3	97.0	99.3	96.4	97.1	99.3	ID	
J	97.4	96.7	96.1	97.4	96.1	96.7	97.4	96.4	96.1	ID

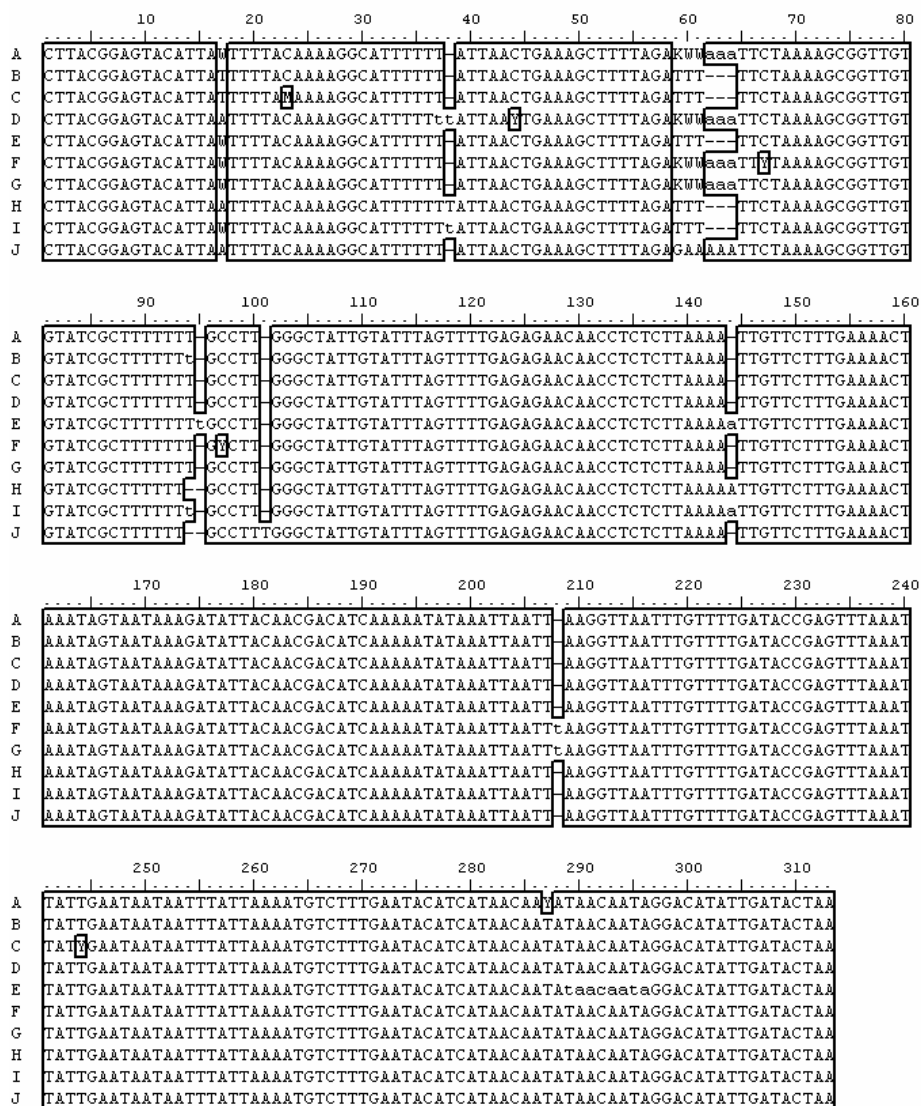


Figure 1

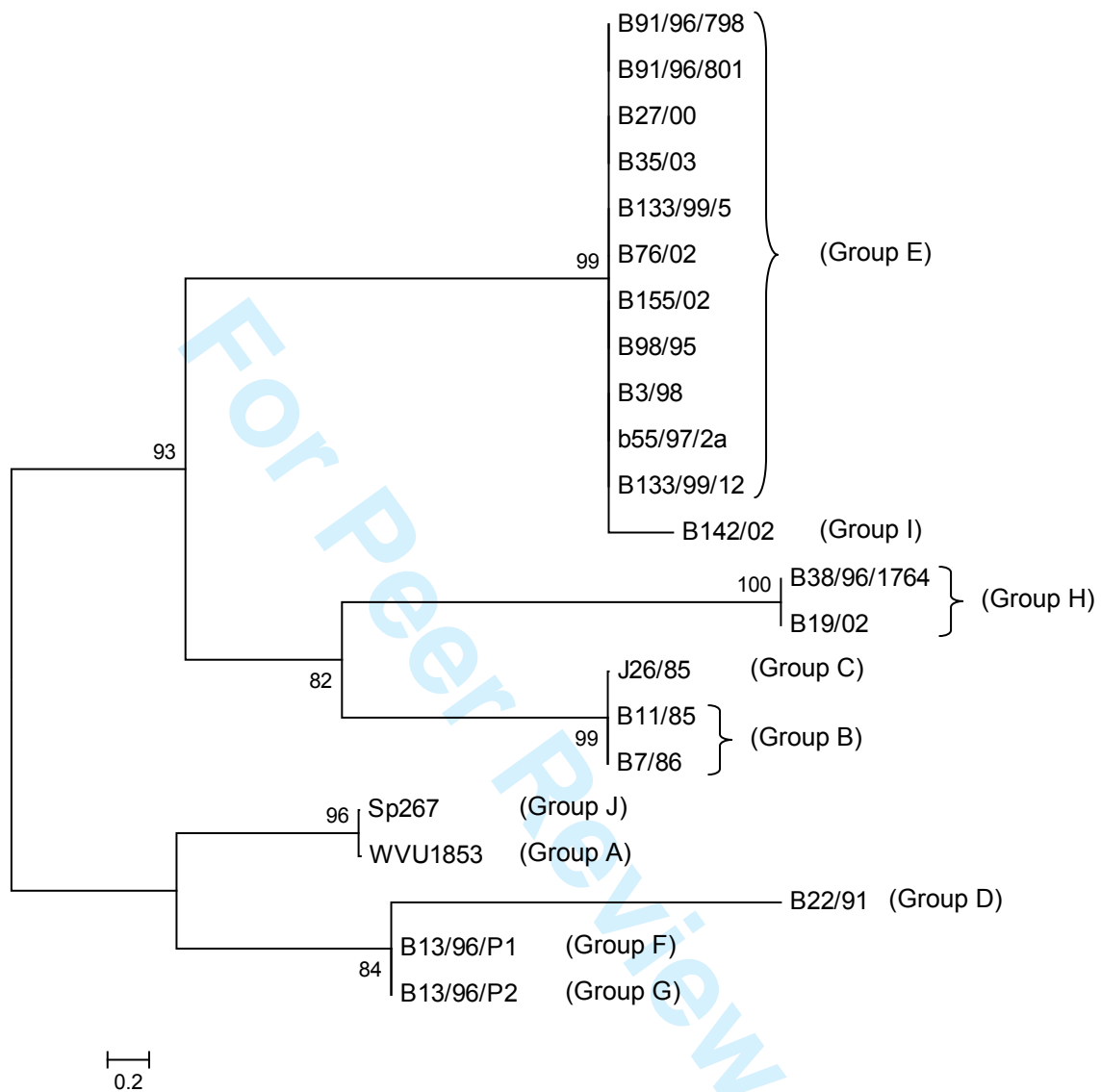


Figure 2.

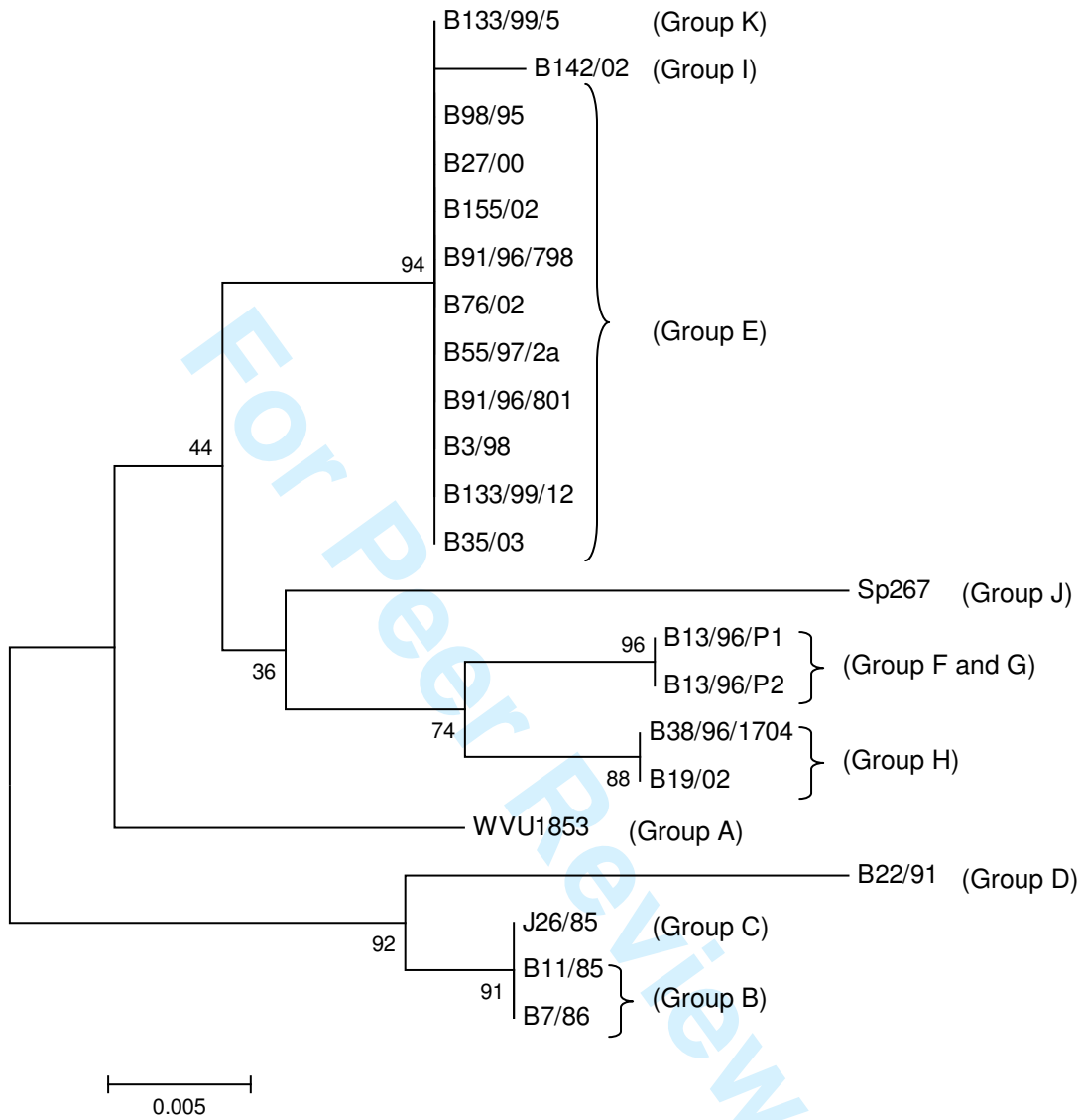


Figure 3.







10 20 30 40 50 60 70 80 90  
 E CTTACGGAGTACATTATTTTACAAAAGGCATTTTTTATTAACTGAAAGCTTTTACATTTTTCIAAAAGCGGTIGIGIATCGCTTTTTT  
 E1 CTTACGGAGTACATTATTTTACAAAAGGCATTTTTTATTAACTGAAAGCTTTTACATTTTTCIAAAAGCGGTIGIGIATCGCTTTTTT  
 E2 CTTACGGAGTACATTATTTTACAAAAGGCATTTTTTATTAACTGAAAGCTTTTACATTTTTCIAAAAGCGGTIGIGIATCGCTTTTTT

100 110 120 130 140 150 160 170 180  
 E tGCCTTGGCCATTTGATTTAGTTTTGAGAGAACAAACCCTCTTAAAAATTGTTCTTTGAAAACIAAATAGTAATAAAGATATTACAACG  
 E1 tGCCTTGGCCATTTGATTTAGTTTTGAGAGAACAAACCCTCTTAAAAATTGTTCTTTGAAAACIAAATAGTAATAAAGATATTACAACG  
 E2 tGCCTTGGCCATTTGATTTAGTTTTGAGAGAACAAACCCTCTTAAAAATTGTTCTTTGAAAACIAAATAGTAATAAAGATATTACAACG

190 200 210 220 230 240 250 260 270  
 E ACATCAAAAAATAAATAAATAAGGTTAATTTGTTTTGATACCGAGTTTAAATTAATGAAATAATAATTTATTAATAAATGICITTTGAATAC  
 E1 ACATCAAAAAATAAATAAATAAGGTTAATTTGTTTTGATACCGAGTTTAAATTAATGAAATAATAATTTATTAATAAATGICITTTGAATAC  
 E2 ACATCAAAAAATAAATAAATAAGGTTAATTTGTTTTGATACCGAGTTTAAATTAATGAAATAATAATTTATTAATAAATGICITTTGAATAC

280 290 300  
 E AATCAATAACAATAAACAATAAGGACATATTGATACTAA  
 E1 AATCAATAACAATAAACAATAAGGACATATTGATACTAA  
 E2 AATCAATAACAATAAACAATAAGGACATATTGATACTAA

10 20 30 40 50 60 70 80 90  
 F CTTACGGAGTACATTATTTTACAAAAGGCATTTTTTATTAACTGAAAGCTTTTACATTTTTCIAAAAGCGGTIGIGIATCGCTTTTT  
 F1 CTTACGGAGTACATTATTTTACAAAAGGCATTTTTTATTAACTGAAAGCTTTTACATTTTTCIAAAAGCGGTIGIGIATCGCTTTTT  
 F2 CTTACGGAGTACATTATTTTACAAAAGGCATTTTTTATTAACTGAAAGCTTTTACATTTTTCIAAAAGCGGTIGIGIATCGCTTTTT

100 110 120 130 140 150 160 170 180  
 F TTTCCTTGGCCATTTGATTTAGTTTTGAGAGAACAAACCCTCTTAAAAATTGTTCTTTGAAAACIAAATAGTAATAAAGATATTACAAC  
 F1 TTTCCTTGGCCATTTGATTTAGTTTTGAGAGAACAAACCCTCTTAAAAATTGTTCTTTGAAAACIAAATAGTAATAAAGATATTACAAC  
 F2 TTTCCTTGGCCATTTGATTTAGTTTTGAGAGAACAAACCCTCTTAAAAATTGTTCTTTGAAAACIAAATAGTAATAAAGATATTACAAC

190 200 210 220 230 240 250 260 270  
 F GACATCAAAAAATAAATAAATAAGGTTAATTTGTTTTGATACCGAGTTTAAATTAATGAAATAATAATTTATTAATAAATGICITTTGAAT  
 F1 GACATCAAAAAATAAATAAATAAGGTTAATTTGTTTTGATACCGAGTTTAAATTAATGAAATAATAATTTATTAATAAATGICITTTGAAT  
 F2 GACATCAAAAAATAAATAAATAAGGTTAATTTGTTTTGATACCGAGTTTAAATTAATGAAATAATAATTTATTAATAAATGICITTTGAAT

280 290 300  
 F AATCAATAACAATAAACAATAAGGACATATTGATACTAA  
 F1 AATCAATAACAATAAACAATAAGGACATATTGATACTAA  
 F2 AATCAATAACAATAAACAATAAGGACATATTGATACTAA

10 20 30 40 50 60 70 80 90  
 G CTTACGGAGTACATTAA TTTTACAAAAGCCATTTTTTTATTAACTGAAAGCTTTTAGATTTTCTAAAAGCGGTIGIGIATCGCTTTT  
 G1 CTTACGGAGTACATTAA TTTTACAAAAGCCATTTTTTTATTAACTGAAAGCTTTTAGATTTTCTAAAAGCGGTIGIGIATCGCTTTT  
 G2 CTTACGGAGTACATTAA TTTTACAAAAGCCATTTTTTTATTAACTGAAAGCTTTTAGATTTTCTAAAAGCGGTIGIGIATCGCTTTT

100 110 120 130 140 150 160 170 180  
 G TTTGCCITGGCTATTIGIATTTAGITTTTGAGAGAACACCCTCTTAAAAATGTCCTTIGAAAACIAAATAGIAATAAAGATATTACAAC  
 G1 TTTGCCITGGCTATTIGIATTTAGITTTTGAGAGAACACCCTCTTAAAAATGTCCTTIGAAAACIAAATAGIAATAAAGATATTACAAC  
 G2 TTTGCCITGGCTATTIGIATTTAGITTTTGAGAGAACACCCTCTTAAAAATGTCCTTIGAAAACIAAATAGIAATAAAGATATTACAAC

190 200 210 220 230 240 250 260 270  
 G GACATCAAAAATATAAATTAATTTAAGGTTAATTTGTTTTGATACCGAGTTTAAATTAATGAAATAAATTTATTAATAAATGTCCTTGAAT  
 G1 GACATCAAAAATATAAATTAATTTAAGGTTAATTTGTTTTGATACCGAGTTTAAATTAATGAAATAAATTTATTAATAAATGTCCTTGAAT  
 G2 GACATCAAAAATATAAATTAATTTAAGGTTAATTTGTTTTGATACCGAGTTTAAATTAATGAAATAAATTTATTAATAAATGTCCTTGAAT

280 290 300  
 G ACATCATAACAATATAACAATAGGACATATTGATACTAA  
 G1 ACATCATAACAATATAACAATAGGACATATTGATACTAA  
 G2 ACATCATAACAATATAACAATAGGACATATTGATACTAA

10 20 30 40 50 60 70 80 90  
 H CTTACGGAGTACATTAA TTTTACAAAAGCCATTTTTTTATTAACTGAAAGCTTTTAGATTTTCTAAAAGCGGTIGIGIATCGCTTTT  
 H1 CTTACGGAGTACATTAA TTTTACAAAAGCCATTTTTTTATTAACTGAAAGCTTTTAGATTTTCTAAAAGCGGTIGIGIATCGCTTTT  
 H2 CTTACGGAGTACATTAA TTTTACAAAAGCCATTTTTTTATTAACTGAAAGCTTTTAGATTTTCTAAAAGCGGTIGIGIATCGCTTTT

100 110 120 130 140 150 160 170 180  
 H GCGTITGGCTATTIGIATTTAGITTTTGAGAGAACACCCTCTTAAAAATGTCCTTIGAAAACIAAATAGIAATAAAGATATTACAACGA  
 H1 GCGTITGGCTATTIGIATTTAGITTTTGAGAGAACACCCTCTTAAAAATGTCCTTIGAAAACIAAATAGIAATAAAGATATTACAACGA  
 H2 GCGTITGGCTATTIGIATTTAGITTTTGAGAGAACACCCTCTTAAAAATGTCCTTIGAAAACIAAATAGIAATAAAGATATTACAACGA

190 200 210 220 230 240 250 260 270  
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 H1 CATCAAAAATATAAATTAATTTAAGGTTAATTTGTTTTGATACCGAGTTTAAATTAATGAAATAAATTTATTAATAAATGTCCTTGAATACA  
 H2 CATCAAAAATATAAATTAATTTAAGGTTAATTTGTTTTGATACCGAGTTTAAATTAATGAAATAAATTTATTAATAAATGTCCTTGAATACA

280 290 300  
 H TCATAACAATATAACAATAGGACATATTGATACTAA  
 H1 TCATAACAATATAACAATAGGACATATTGATACTAA  
 H2 TCATAACAATATAACAATAGGACATATTGATACTAA

