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Evaluation of Amplified Fragment Length Polymorphism for Differentiation of Avian Mycoplasma Species

Yang Hong,[1] Maricarmen García,[1]* Sharon Levisohn,[2] Inna Lysnyansky,[2] Victoria Leiting,[1] P. H. M. Savelkoul,[3] and S. H. Kleven[1]

Department of Avian Medicine, College of Veterinary Medicine, University of Georgia, Athens, Georgia[1]; Division of Avian & Aquatic Diseases, Kimron Veterinary Institute, Bet Dagan, Israel[2]; and Department of Medical Microbiology and Infection Control, University Hospital Vrije Universiteit, Amsterdam, The Netherlands[3]

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Amplified fragment length polymorphism (AFLP) was used for typing avian mycoplasma species. Forty-four avian mycoplasma strains were successfully typed into eight distinct groups, with each representing a different species. Homology of AFLP patterns of 35% or less was used as a cutoff value to differentiate avian mycoplasma strains into different species.

Some species of avian mycoplasma are important disease factors which adversely affect the commercial poultry industry in the United States. Avian mycoplasmas are very small prokaryotes devoid of cell walls. They tend to grow very slowly on a protein-rich medium containing 10 to 15% added animal serum and are rather resistant to certain antibiotics which are frequently employed in medium to retard growth of contaminant bacteria and fungi (4). *Mycoplasma gallisepticum* is the most pathogenic avian mycoplasma species, causing chronic respiratory diseases in chickens and infectious sinusitis in turkeys (11). When the infection becomes systemic, *M. synoviae* can also cause infectious synovitis in chickens and turkeys (5). *M. iowae* infection leads to reduced hatchability and embryo mortality in turkeys.

Condemnation of the infected flocks and reduction in feed conversion and egg production are the major factors related with economic losses. However, nonpathogenic species such as *M. gallinarum* and *M. gallinaceum* are often isolated and must be differentiated from pathogenic species. Amplified fragment length polymorphism (AFLP) has been extensively tested by Kokotovic et al. for typing mycoplasma species isolated from food animals such as cattle (9), swine (8), and goats (6) and from humans (7). The major advantage of this technique compared to other molecular typing methods is that it requires a relatively small amount of DNA and has great discriminatory power and reproducibility (15). So far, the system has not been evaluated for typing avian mycoplasma species. The objective of this study was to apply AFLP for differentiating avian mycoplasma species.

A total of 44 strains of avian mycoplasma representing eight different species were used in this study (Table 1). They were obtained from the depository at the Poultry Diagnostic and Research Center in Athens, Ga. Mycoplasma strains were cultured in Frey's medium with 12% swine serum (2). Unlike Kokotovic's procedure (7), the DNA template in this study was prepared by a simple boiling method described by Fan et al. (2). Mycoplasma culture (1.5 ml) harvested at late log phase was used for DNA extraction. DNA was stored at −20°C until used.

Genomic DNA digestion, adapter ligation, and selective amplification were performed following the procedure described by Kokotovic et al. (7). Restriction endonucleases BglII and MfeI were used in the digestion of genomic DNA. The MFE1 primer used in this study, as in Kokotovic's study, was modified by adding a selective nucleotide A at its 3′ end to increase the selectivity of the amplification reaction and to obtain better banding pattern resolution.

Fragment detection was carried on an ABI 310 automatic sequencer (ABI Applied Biosystems, Foster City, Calif.). A mixture consisting of 2.0 μl of PCR products, 12.0 μl of 100% deionized formamide, and 0.5 μl of GeneScan 500 ROX size standard (ABI Applied Biosystems, Foster City, Calif.) was heated to 95°C for 5 min and quickly chilled on ice before electrophoresis on the machine. Fragment size determination and pattern analysis were performed by using GeneScan 3.1 fragment analysis software (ABI Applied Biosystems). ABI chromatograms were converted into schematic gel images with GelCompar II 3.5 (Applied Math Inc., Austin, Texas). Background subtraction and data normalization were subsequently conducted. Cluster analysis was performed using the Pearson correlation and unweighted pair group methods with average linkages.

AFLP analysis in this study provided an optimal separation and a uniform sizing of the amplified fragments. Fragments of between 75 and 500 bp were used in numerical and cluster analysis for species differentiation. We found that *M. gallisepticum* strains had the highest banding pattern complexity, consisting of about 90 AFLP fragments, while *M. meleagridis* profiles had the lowest complexity, having approximately only 20 amplified fragments. Both *M. gallinarum* and *M. iowae* strains contained about 50 fragments. About 60, 40, and 30 fragments were generated for *M. gallinaceum*, *M. pullorum*, and *M. synoviae*, respectively. *M. imitans* had 70 fragments in its AFLP banding pattern. Reproducibility of AFLP analysis has been extensively tested by Kokotovic et al. (7). In this study, AFLP

* Corresponding author. Mailing address: Department of Avian Medicine, College of Veterinary Medicine, University of Georgia, 953 College Station Rd., Athens, GA 30602. Phone: (706) 542-5656. Fax: (706) 542-5630. E-mail: mcgarcia@uga.edu.

TABLE 1. Mycoplasma strains used in this study

Organism	Strain	Isolated from:	Source or history
M. gallisepticum	K703	Chicken	Atypical field isolate
	K730	Chicken	Atypical field isolate
	K4110A	Turkey trachea	Field isolate
	K4110B	Turkey trachea	Field isolate
	K4492A-1	Chicken trachea	Field isolate
	Ts-11	Chicken	Vaccine (Merial)
	6/85	Unknown	Vaccine (Intervet)
	F	Chicken trachea	Vaccine (Schering-Plough)
	R	Chicken trachea	Pathogenic strain
M. synoviae	K1968	Turkey joint	Pathogenic field isolate
	K4822B	Turkey trachea	Field isolate
	K4822C	Turkey trachea	Field isolate
	K4822D	Turkey trachea	Field isolate
	K5001-2	Chicken trachea	Field isolate
	K5001-3	Chicken trachea	Field isolate
	K5001-5	Chicken trachea	Field isolate
	WVU-1853	Chicken joint	Type strain
M. iowae	695	Turkey air sac	Type strain
	693	Turkey joint	Reference strain
	DK-CPA	Turkey embryo	Reference strain
	DNA-O	Turkey air sac	Reference strain
	L3-10B	Turkey yolk sac	Reference strain
	DRA-O	Turkey air sac	Reference strain
	K5378-10	Turkey embryo	Field isolate
	K5413-2	Turkey embryo	Field isolate
	K5521-5	Turkey embryo	Field isolate
M. meleagridis	K4766-1	Turkey trachea	Field isolate
	K4766-2	Turkey trachea	Field isolate
	K5428A-11	Turkey trachea	Field isolate
	RY39A	Turkey	Pathogenic strain
	CA-6	Turkey	Reference strain
	E-2	Turkey embryo	Hemagglutinating strain
M. gallinarum	LPG-16	Chicken trachea	Reference strain
	K5446A-15	Chicken trachea	Field isolate
	SB.B	Chicken trachea	Field isolate
	1504	Chicken trachea	Reference strain
M. gallinaceum	SA	Chicken	Reference strain
	R85A	Chicken	Reference strain
	Tully DD	Chicken	Reference strain
	S-594TT	Chicken	Reference strain
M. pullorum	K285-496A	Chicken	Field isolate
	K285D-2403	Chicken	Field isolate
	y96	Chicken	Reference strain
M. imitans	4229	Duck	ATCC type strain

procedure was repeated three times on three randomly chosen strains from each species and highly reproducible results were obtained. Minor changes in band intensities existed but were insignificant for determining identity of strains (data not shown).

On the basis of cluster analysis, we chose the 35.0% linkage level (percent homology) as a cutoff value for discriminating mycoplasma strains at the species level (Fig. 1). AFLP data revealed eight distinct groups (I to VIII), each consisting of strains belonging to a single species. All strains within each group had different AFLP patterns, some of which were nearly identical. To validate this clustering method, we calculated error flags (not shown in the dendrogram) representing the value of the mean linkage level plus standard deviation for those groups of strains as indicated in the following paragraphs. The 35.0% cutoff line did not cross any of those error flags.

M. pullorum, *M. gallinarum*, and *M. gallinaceum* strains showed high AFLP pattern heterogeneity, with linkage levels of 47.8% ± 4.5% (mean plus standard deviation) (group III), 48.0% ± 9.0% (group VI), and 51.4% ± 6.8% (group IV), respectively. Our study is the first to report the genetic heterogeneity among those saprophytic avian mycoplasma species, though a larger number of strains, as well as other restriction enzymes, needs to be tested to make final conclusions.

On the other hand, *M. iowae* and *M. meleagridis* strains

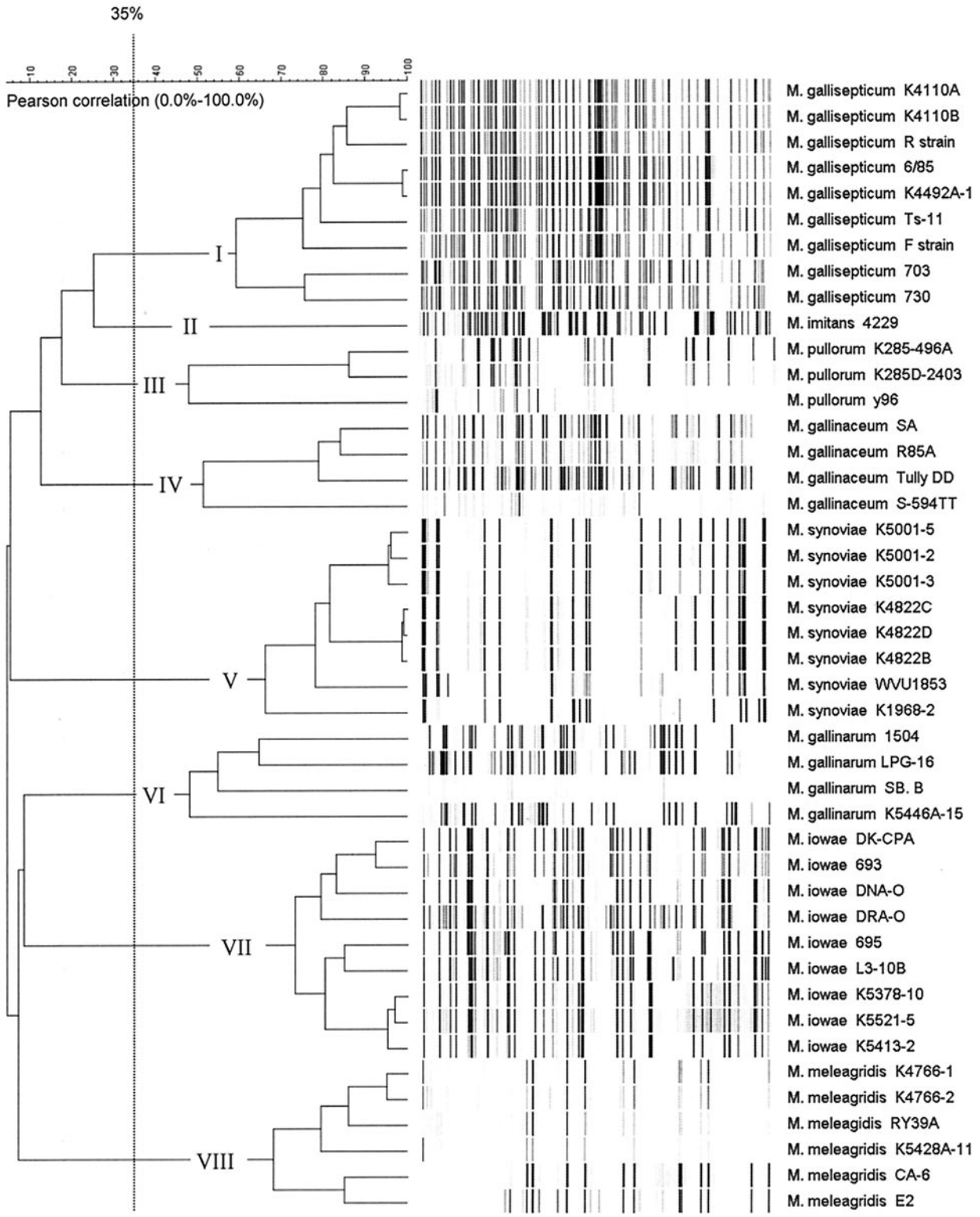

FIG. 1. AFLP fingerprints of avian mycoplasma species. The dendrogram was constructed using Pearson correlation and the unweighted pair group method with average linkages. The eight avian mycoplasma species groups generated at a 35.0% linkage level cutoff point are indicated.

showed high homogeneity in their AFLP profiles and clustered at linkage levels of 73.2% ± 6.2% (group VII) and 68.0% ± 7.8% (group VIII), respectively. Previous studies revealed that although heterogeneity in serological responses was observed for *M. iowae* (10), less-variable protein profiles and random amplified polymorphic DNA (RAPD) patterns were obtained for different strains (3, 14). Among pathogenic avian mycoplasmas, *M. gallisepticum* strains revealed the widest intraspecies heterogeneity by AFLP analysis, with a linkage level of 59.2% ± 2.2% (group I). The genetic variation of this species has been documented by several other molecular typing techniques, such as pulse field gel electrophoresis (12), random amplified polymorphic DNA (2, 12), restriction fragment length polymorphism (13), and Southern blotting (17). *M. synoviae* strains, with a linkage level of 66.1% ± 3.0% (group V), exhibited more genetic homogeneity than *M. gallisepticum*, and the same conclusion was made using an rRNA gene hybridization test conducted by Yogev et al. (16).

M. imitans shared many phenotypic properties with *M. gallisepticum* but had low genetic homology with *M. gallisepticum* in a DNA-DNA hybridization study (1). In this study, the two species were typed into related groups and linked at the 25.3% homology level.

As determined on the basis of these results, AFLP can be used as an additional confirmatory tool for identification of avian mycoplasma species. This can be achieved by setting up a database for reference strains; such a database was partially created in this study and can be easily expanded. Our follow-up studies will focus on using AFLP for typing avian mycoplasma strains within each species.

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