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Evaluation of random amplified polymorphic DNA and multiple-locus variable number tandem repeat analyses for *Mycoplasma cynos*

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ABSTRACT

Mycoplasma spp. can cause diseases of the respiratory system as well as urogenital infections, infertility, and anemia. The members of this genus have a low G + C content compared to other bacteria. Because primers used in the random amplified polymorphic DNA (RAPD) technique are only 10 bp long and have high GC content, this method can be inadequate for genotyping *Mycoplasma* spp. isolates. The aim of this study was to develop and evaluate multiple-locus variable number tandem repeat analysis (MLVA) and two-primer RAPD (TP-RAPD) procedures for subtyping *Mycoplasma cynos* isolates.

A total of 55 *M. cynos* isolates obtained from 162 bronchoalveolar lavage fluid samples from shelter and pet dogs were used in this study. Seventy-four tandem repeat regions were detected in the *M. cynos* genome, and two of these loci were determined to be suitable and used for development of the MLVA scheme. The results of variable number tandem repeat (VNTR) analysis and TP-RAPD-PCR were compared with RAPD-PCR. The discriminatory power of TP-RAPD-PCR (Hunter-Gaston diversity index [HGDI] = 0.84) was higher than those of RAPD-PCR (HGDI = 0.727), VNTR1 (HGDI = 0.8), and VNTR3 (HGDI = 0.757). We observed that the TP-RAPD-PCR and MLVA methods provide clearer data and are more successful in determining genetic diversity, in contrast to the RAPD-PCR method for this species.

1. Introduction

Canine infectious respiratory disease (CIRD) is a major cause of morbidity and an important welfare issue for kenneled dog populations worldwide (Appel and Binn, 1987; Randolph et al., 1993). Several *Mycoplasma* spp., including *Mycoplasma canis*, *Mycoplasma cynos*, and *Mycoplasma edwardii,* have been isolated from the lower respiratory tracts of dogs with clinical disease (May and Brown, 2009; Mitchell et al., 2017). Additionally, *M. cynos* is the only species of the genus *Mycoplasma* commonly associated with canine respiratory disease (May and Brown, 2009; Nagatomo et al., 2001; Rosendal, 1972). In canine pulmonary disease, the role of *Mycoplasma* spp. as primary or secondary pathogens has been discussed in several studies (Chandler and Lappin, 2002, Randolph et al., 1993); however, it is difficult to isolate this agent (Chalker et al., 2004).

The majority of canine species affecting dogs have been included in the Hominis taxonomic group of the genus *Mycoplasma*, except *Mycoplasma haemocanis* and *Ureaplasma canigenitalium*, and their diversity is reflected in the G + C mol% content of each species, which ranges from 23.8 to 36.0. Similarly, the genome size of the Mollicutes varies from 606 to 1650 kb (Chalker, 2005). It has also been reported that *M. cynos* strains possess as an A + T mol% of 74–76.8 compared to G + C mol% of 25.8–26 (Razin and Tully, 1995; Walker et al., 2013). This relationship between mutation-rate ratio and gene GC content suggests that in genes with low GC content, the mutational rate of AT is orders of magnitude higher than the mutational rate of GC (Brocchieri, 2013).

In a previous report, the genetic similarity of the *M. cynos* type strain and 14 isolates with respiratory disease was investigated using Pulsed-Field Gel Electrophoresis (PFGE) and the Random Amplified Polymorphic DNA (RAPD) method, and a larger strain typing study of more isolates was required to consolidate these observations (Mannering et al., 2009). Although PFGE is currently considered to be the gold standard for subtyping bacteria, especially in foodborne disease, it requires specific equipment, technical skills, and is time consuming and expensive (Sintchenko et al., 2012). RAPD technology has

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Table 1
PCR primers, cycle conditions, and product sizes.

Primer	Sequence (5′ → 3′)	Number of cycles	PCR product (bp)
MGSO[a]	TGC ACC ATC TGT CAC TCT GTT AAC CTC	5′ 94 °C, 35 (30″ 94 °C; 30″ 56 °C; and 30″ 72 °C), 10′ 72 °C	1013
GPF[a]	GCT GGC TGT GTG CCT AAT ACA		
Myc1[b]	ACCGCCCGTCACACCA	5′ 95 °C, 35 (45″ 95 °C; 30″ 55 °C; and 20″ 72 °C), 5′ 72 °C	227
M cynos[b]	GATACATAAACACAACATTATAATATTG		
Hum 4[c]	ACGGTACACT	5′ 94 °C, 40 (15″ 94 °C; 60″ 37 °C; and 90″ 72 °C), 7′ 72 °C	Variable
TPRAPD1[c]	CCAGTCTAGCGGCCTTGGTC	40 (60″ 95 °C; 60″ 58.9 °C; and 90″ 72 °C), 7′ 72 °C	Variable
TPRAPD2[c]	GCTTTTGCGCGGTTTTTG		
VNTR1F[c]	CAACGGACATTGTTGAAGC	40 (3′ 95 °C; 60″ 53.2 °C; and 90″ 72 °C)	Variable
VNTR1R[c]	TCTTCCACCACTCGACATC		
VNTR3F[c]	TCGACGTGCATTCAACTCACT	40 (3′ 95 °C; 60″ 55.8 °C; and 90″ 72 °C)	Variable
VNTR3R[c]	CAATGCACGGTTTGCATCATC		

All PCRs were performed using 5 μL 5× FIREPol®Master Mix (Solis Biodyne, Estonia), 20 pmol of each primer, and DNA template (100 ng/μL), and 1 μL water (negative control). Positive control DNA was used in each PCR series. PCR products were resolved by electrophoresis on 10 cm 2% agarose gels at 60 mA for 1.5 h, stained with ethidium bromide and visualized under UV illumination.

[a] 1 kb DNA ladder (Thermo Scientific, SM0313).

[b] 100 bp.

[c] 50 bp DNA ladder (Thermo Scientific, SM0373) was used for comparison of DNA sizes.

Table 2
Characterization of the VNTR regions.

Locus	Flanking region (5′)		Sequence of repeat unit	Flanking region (3′)		Location in C142	Allele number
	Length (bp)	Partial sequence		Length (bp)	Partial sequence		
VNTR1	161	atggcctttctttgaccc	tcagctttaagtatt	131	gatttttgaccttcagc	157043–157129	x-(161 + 131)/15
VNTR3	228	gctcaatcatcagtgcatgt	tgatgaaaaataatacaattac	131	gacgtaattatacttata	633942–638381	x-(228 + 131)/21

distinct advantages such as availability, efficiency, and low expense (Kumari and Thakur, 2014). Particular disadvantages are the production of non-specific bands (Sudan et al., 2017) and use of primers only 10 bp long and with high GC content (Kumari and Thakur, 2014). Additionally, it was reported that as the number of primers used also increases, the number of bands obtained also increases (Rivas et al., 2001). Although RAPD has been used as an easily performed and highly discriminatory test to type strains of several *Mycoplasma* species, it lacks reproducibility, making a comparison of new isolates only possible by reanalyzing all isolates in a single experiment (Stakenborg, 2005). Mannering et al. (2009) reported that the PFGE and RAPD genetic typing methods were in basic agreement and showed that many of the *M. cynos* isolates tested were highly similar.

The procedure named two-primer RAPD (TP-RAPD) was proposed to obtain fingerprints of bacteria using two primers in PCR. Compared to RAPD-PCR, The relatively high annealing temperature (50 °C) avoids problems of reproducibility as described by other authors (Cusick and O'Sullivan, 2000). TP-RAPD fingerprinting can be applied to bacterial taxonomy, ecological studies, and the detection of new bacterial species. Its advantages include high sensitivity, reliability, and reproducibility, as well as rapid turnaround time; it is also applicable for a large number of microorganisms (Rivas et al., 2001, 2002).

The Multiple- Locus Variable Number Tandem Repeat Analysis (MLVA) method has also been used for subtyping of bacteria, and this method has proven to be a rapid method that can be easily standardized between laboratories (Sintchenko et al., 2012). Variable number tandem repeat (VNTR) analysis, a method based on tandem repeat polymorphisms at multiple loci, has been successfully applied to many bacterial species (Pourcel et al., 2009).

In this study, we compared the use of RAPD and new PCR-based techniques (TP-RAPD, and two VNTR regions) with respect to discriminatory power, reproducibility, and ease of performance for genotyping of *M. cynos* strains.

2. Materials and methods

2.1. M. cynos isolates

Several species of mycoplasma have been isolated from the lower respiratory tract of both healthy and diseased dogs (Chalker, 2005). Therefore, bronchoalveolar lavage (BAL) samples were used in this study. BAL samples were collected from 126 stray dogs kept in shelter houses in Konya Municipality, Turkey, as well as 36 pet dogs from the Faculty of Veterinary Medicine, Selcuk University, Konya. This research was approved (2014/59) by the Ethics Committee of the Faculty of Veterinary Medicine at the University of Selcuk in Konya, Turkey. All animals included in this study showed at least one of the following clinical signs of respiratory infection: fever, cough, nasal discharge, increased respiratory rate, and abnormal bronchial sounds. The BAL samples were taken by passing a nasotracheal tube and then infusing 10 mL of sterile saline solution under local anesthesia with lidocaine. Standard medium used for isolation of large Mycoplasma colonies was prepared according to the Hayflick protocol (Hayflick, 1965). Briefly, it consisted of 7 parts of PPLO agar (Thermo Fisher Scientific™ Oxoid™, USA), 2 parts of sterile horse serum, 1 part of 25% yeast extract, 0.05 mg thallium acetate, and 1000 units of penicillin G per mL. 100 μL of the BAL samples were spread onto the medium, and incubated at 37 °C in 95% nitrogen and 5% CO_2 for 7 days (Hayflick, 1965). Suspicious *M. cynos* isolates were confirmed by a PCR method as previously described (Chalker et al., 2004). *M. cynos* was cultured on mycoplasma medium, and cultures of the single-cloned *M. cynos* isolates were stored at −80 °C. The type strain *M. cynos* H381 NCTC10142 was used as a reference strain.

2.2. DNA extraction

Chromosomal DNA from *M. cynos* strains was extracted from a colony using the GenElute™ gel extraction kit (Sigma-Aldrich, Munich, Germany) according to a previously published method (Sakmanoglu et al., 2017). The DNA samples were stored at −20 °C until PCR

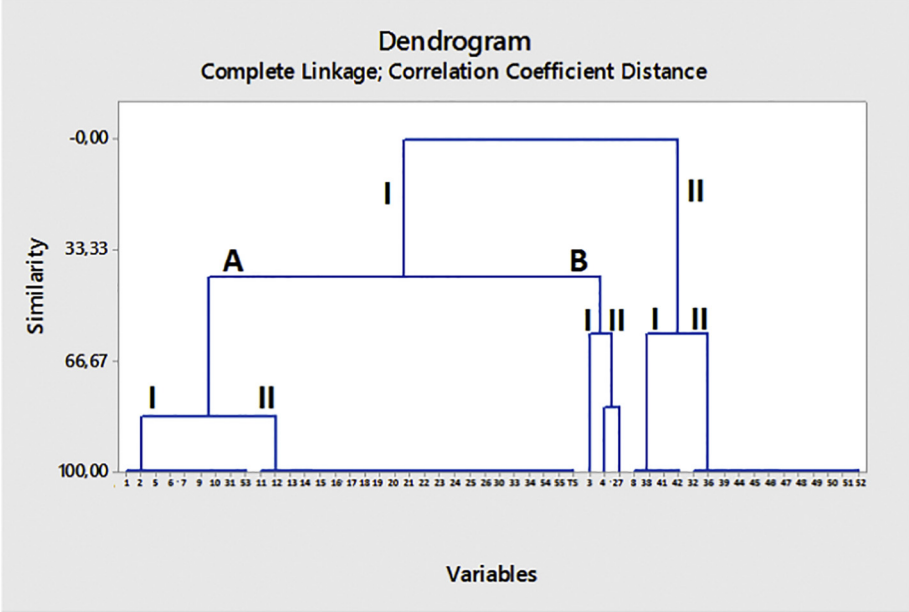

Fig. 1. Similarity analysis of the profiles produced by RAPD cluster analysis. 1–55, *Mycoplasma cynos* isolates; TS, Type strain.

analysis.

2.3. Identification by PCR

Firstly, MGSO and GPF primers were used to confirm *Mycoplasma* spp. isolates obtained from BAL samples as previously described (Lierz et al., 2007) (Table 1). Then, Myc1 and *M. cynos* primers were used to amplify the 16S/23S rRNA intergenic spacer regions according to a published method (Chalker et al., 2004; Hong and Kim, 2012). The amplification reaction was performed as described in Table 1.

2.4. Genotyping

2.4.1. RAPD-PCR

The single Hum4 primer was used to generate RAPD profiles of *M. cynos* (Hotzel et al., 1998) (Table 1). The sizes of PCR products were determined by agarose gel electrophoresis in comparison to known standards, and the number of randomly amplified bands were determined. The agarose gel was prepared as described in Table 1.

2.4.2. TP-RAPD-PCR

Alternate primers were necessary for the genotyping of *M. cynos* isolates in this study, because the universal 8F and 1491R primers that had previously been used in TP-RAPD were utilized for PCR fingerprinting of GC-rich rhizobia. Although the TPRAPD1 and TPRAPD2 primers were predicted to give a single amplicon based on the complete genome sequence of *M. cynos* C142 (GenBank Accession number NC-019949.1), when evaluated empirically, they were found to be suitable for TP-RAPD, in that multiple bands were obtained and that the profile was strain variable. The Tandem Repeats Finder software v.4.08 (http://tandem.bu.edu/trf/trf.html) (Benson, 1999), was used to evaluate the ratio of GC and AT percentages in the primers and genome. Validation of PCR amplification of the loci was performed for the development of TP-RAPD of *M. cynos* isolates. The detection limit was 10 ng/μL extracted DNA for the type strain and other strains. The TP-RAPD-PCR reaction was performed according to the conditions listed in Table 1. Agarose gel electrophoresis was used to determine the sizes of PCR products as described in Table 1.

2.4.3. MLVA

Validation of PCR amplification of the loci was performed for development of MLVA of *M. cynos* isolates. The PCR assays for the 2 MLVA loci (VNTR1 and VNTR3) were optimized for genotyping of *M. cynos*. The detection limit was 10 ng/μL of extracted DNA for *M. cynos* H381 NCTC10142 and the other strains.

In total, 74 candidate VNTR regions were obtained as a result of a whole genome database search of *M. cynos* C142 (GenBank Accession number NC_019949.1) using the Tandem Repeats Finder software (Benson, 1999). Among them, 4 regions appeared suitable for MLVA according to period and consensus size. Two loci (VNTR1, VNTR3) showed robust amplification and sufficient polymorphism for use in MLVA typing. Additionally, one tandem repeat (VNTR3) was detected in the *M. cynos* rpoB gene encoding RNA polymerase beta subunit.

The primer sets were designed within 250 bp from the end of VNTR regions to minimize the length of flanking regions, and the number of repeat units is shown in Table 2. MLVA was performed with VNTR and VNTR3 primers according to the cycle conditions described in Table 1. The numbers of repeated units were calculated by using the model size designed after determining the length of the PCR products using agarose gel electrophoresis (Table 1).

2.5. Data analysis

RAPD-PCR, TP-RAPD-PCR and MLVA were compared by 3 procedures: Unweighted Pair Group Method with Arithmetic mean (UPGMA) using Pearson correlation coefficient was detected on the distance between two data points that were connected (Sokal and Michener, 1958), phylogenetic tree was constructed using the Hierarchical Cluster Analysis Method (IBM SPSS Statistic 23 Programme) and the Hunter-Gaston Discriminatory Index (HGDI) for the strain typing scheme was calculated (Hunter and Gaston, 1988) with 95% confidence intervals by programme (Grundmann et al., 2001).

3. Results

3.1. M. cynos isolates

Amplification products to determine whether isolates were *Mycoplasma* spp. or *M. cynos* were 1013 bp and 227 bp, respectively. A total of 55 candidate *Mycoplasma* spp. were isolated from BAL samples, and all isolates were confirmed as a *Mycoplasma* spp. by PCR. All 55 isolates, including 52 (41.26%) from shelter dogs and 3 (8.33%) from

Table 3
Mycoplasma cynos isolate source and genetic typing groups by different methods.

Dog	*M. cynos* isolate	RAPD-PCR group	TP-RAPD group	VNTR1 group	VNTR3 group
Type strain	*M. cynos* H381	I-A-II	I-B-I	II-B-II	II-A-II
	NCTC10142				
Shelter	1	I-A-I	II-II	I-A	I-A
Shelter	2	I-A-I	II-I	II-A-I	II-A-I
Shelter	3	I-B-I	I-A-II	II-A-I	I-A
Shelter	4	I-B-II	I-B-I	I-A	I-A
Shelter	5	I-A-I	I-A-I	II-A-I	II-A-I
Shelter	6	I-A-I	I-A-I	I-A	I-A
Shelter	7	I-A-I	I-A-I	II-B-I	II-A-I
Shelter	8	II-I	I-A-I	II-A-II	I-A
Shelter	9	I-A-I	I-A-I	II-B-I	I-A
Shelter	10	I-A-I	I-A-I	II-B-II	I-A
Shelter	11	I-A-II	I-B-I	II-B-II	I-B
Shelter	12	I-A-II	I-B-I	II-B-II	II-A-I
Shelter	13	I-A-II	I-B-I	II-B-II	I-A
Shelter	14	I-A-II	I-B-I	II-B-II	I-A
Shelter	15	I-A-II	I-B-I	II-B-II	I-B
Shelter	16	I-A-II	I-B-I	II-B-II	II-A-I
Shelter	17	I-A-II	I-B-I	II-B-II	II-A-II
Shelter	18	I-A-II	I-B-I	II-B-II	II-B-I
Shelter	19	I-A-II	I-B-I	II-B-II	II-B-II
Shelter	20	I-A-II	I-B-I	II-B-II	II-B-II
Shelter	21	I-A-II	I-B-I	II-B-II	II-B-II
Shelter	22	I-A-II	I-B-I	II-B-II	II-B-II
Shelter	23	I-A-II	I-B-I	II-B-II	I-B
Shelter	24	I-A-II	I-B-I	II-B-II	II-B-II
Shelter	25	I-A-II	I-B-I	II-B-II	II-B-II
Shelter	26	I-A-II	I-B-I	II-B-II	II-B-II
Shelter	27	I-B-II	I-B-I	II-B-II	II-B-II
Shelter	28[a]	–	I-B-I	II-B-II	I-A
Shelter	29[a]	–	I-B-I	II-B-II	II-B-II
Shelter	30	I-A-II	I-B-I	II-B-II	II-B-I
Shelter	31	I-A-I	I-B-I	I-A	II-A-I
Shelter	32	II-II	I-B-I	II-B-I	II-A-II
Shelter	33	I-A-II	I-B-I	II-A-II	II-A-II
Shelter	34	I-A-II	II-I	II-B-II	II-A-II
Shelter	35[a]	–	II-I	II-B-I	II-A-II
Shelter	36	II-II	II-I	II-B-I	II-A-II
Shelter	37[a]	–	II-I	II-A-I	II-B-I
Shelter	38	II-I	II-I	II-B-I	II-B-I
Shelter	39	II-II	II-I	II-A-I	II-B-II
Shelter	40[a]	–	II-I	II-B-I	II-B-II
Shelter	41	II-I	II-I	II-B-II	II-B-II
Shelter	42	II-I	II-I	II-B-I	II-A-I
Shelter	43[a]	–	II-I	II-B-I	II-A-I
Shelter	44	II-II	I-B-I	II-B-II	II-A-I
Shelter	45	II-II	II-I	II-B-II	II-A-I
Shelter	46	II-II	I-A-II	II-B-II	II-A-I
Shelter	47	II-II	I-A-II	II-A-I	I-A-I
Shelter	48	II-II	I-A-II	II-B-II	I-A-I
Shelter	49	II-II	II-I	II-B-I	I-A
Shelter	50	II-II	II-I	II-B-II	I-B
Shelter	51	II-II	II-I	II-B-II	I-A
Shelter	52	II-II	II-I	II-A-II	I-A
Pet	53	I-A-I	I-B-I	II-B-I	II-B-II
Pet	54	I-A-II	I-B-I	II-B-I	II-B-II
Pet	55	I-A-II	I-B-I	II-B-II	II-B-I

[a] The RAPD profiles of 28, 29, 35, 37, 40 and 43 isolates were not detected by this procedure.

pet dogs, were identified as *M. cynos* by PCR.

3.2. RAPD-PCR

According to the results, 6 different profiles were obtained from 49/55 *M. cynos* isolates. RAPD profiles from 28, 29, 35, 37, 40, and 43 were not obtained by this procedure. The profiles consisted of 1–12 bands that ranged in size between ~ 110 and 1680 bp. Two main groups were determined by cluster analysis. Group I contained the type strain and 33 isolates (1–7, 9–27, 30, 31, 33, 34 and 53–55), and was a genetically homogeneous group with at least 91.42% similarity. High percentages of the *M. cynos* isolates distinguished based on the origin of the dogs were found in the same cluster (sub-group A of cluster I), 65.21%, (n = 30) for the shelter dogs and 100% (n = 3) for the pet dogs (Fig. 1) (Table 3). The isolates (8, 32, 36, 38, 39, 41, 42, 44–52) were detected as a Group II of which was a genetically homogeneous group with at least 74.92% similarity. Jaccard's coefficient of similarity was determined to be 0.778 by UPGMA, and HGDI was calculated to be 0.727.

3.3. TP-RAPD-PCR

All *M. cynos* isolates were classified as a 6 different profiles which consisted of 2–15 bands that ranged in size between ~ 140 and 3050 bp, and profiles were grouped by similarity into two groups using cluster analysis. The type strain and 38 isolates (3–33, 44, 46–48, and 53–55) took part in Group I of which was a genetically homogeneous group with at least 91.42% similarity. High percentages of the all isolates distinguished based on the origin of the dogs were found in the same cluster (sub-group B of cluster I), 67.3%, (n = 35) for the shelter dogs and 100% (n = 3) for the pet dogs (Fig. 2) (Table 3). Group II, which contained the isolates (1, 2, 34–43, 45, 49–52), was a genetically homogeneous group with at least 74.95% similarity. Jaccard's coefficient of similarity was determined to be 0.844 by UPGMA, and HGDI was calculated to be 0.84.

3.4. Development of the MLVA method for M. cynos

The number of repeats in the VNTR regions was 5.4–33.86 and 1.95–3.85 in VNTR1 and VNTR3, respectively. Seven and 6 profiles were obtained from VNTR1 and VNTR3, respectively.

According to cluster analysis results of VNTR1, two broad groups were obtained from isolates, and the profiles consisted of 1–8 bands that ranged in size between ~ 370 and 800 bp. Group II contained the type strain and 51 isolates (1–3, 5, 7–30 and 32–55), and the similarity of the group was determined as a least 79.8%. High percentages of the *M. cynos* isolates distinguished based on the origin of the dogs were found in the same cluster (sub-group B of cluster II), 92.3%, (n = 48) for the shelter dogs and 100% (n = 3) for the pet dogs (Fig. 3) (Table 3). Group I comprised the isolates (1, 4, 6 and 31) which was a genetically homogeneous group with at least 79.8% similarity. Jaccard's coefficient of similarity was determined to be 0.829 by UPGMA. HGDI was calculated to be 0.8.

For VNTR3, six profiles consist of 1 to 7 bands that ranged in size between ~ 400 and 440 bp, and isolates were classified two main groups by cluster analysis. The type strain and 42 isolates (2, 5, 7, 12, 16, 17–22, 24–27, 29–48 and 53–55) were found in Group II of which was a genetically homogeneous group with at least 83.5% similarity. A high percentage of the *M. cynos* isolates based on the origin of dogs were found in the same cluster (sub-group A of cluster II), 75%, (n = 39) for the shelter dogs and 100%, (n = 3) for the pet dogs (Fig. 4) (Table 3). Group I contained the isolates (1, 3, 4, 6, 8–11, 13–15, 23, 28, 49 and 50–52) which were a genetically homogeneous group with at least 74.92% similarity. Jaccard's coefficient of similarity was determined to be 0.804 by UPGMA, and HGDI was calculated to be 0.757.

4. Discussion

The RAPD-PCR efficiency may be variable due to the primer annealing with low specificity to regions on the genome. TP-RAPD-PCR method is more sensitive than RAPD-PCR because of the high annealing temperature and the use of two primers. In MLVA, tandem repeat number and consensus sizes play a predominant role in bacterial genotyping. Therefore, there may be group differences between the isolates

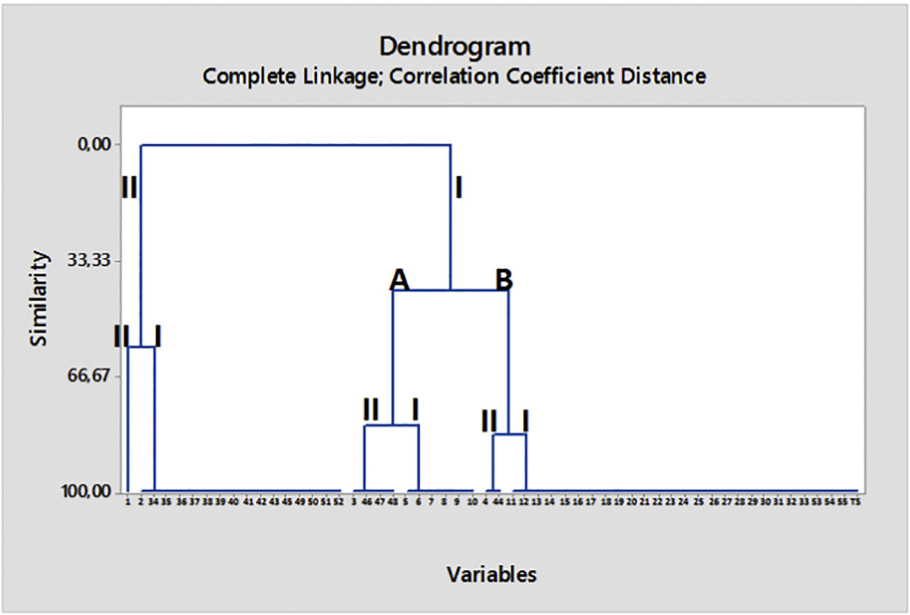

Fig. 2. Similarity analysis of the profiles produced by TP-RAPD cluster analysis. 1–55, *Mycoplasma cynos* isolates; TS, Type strain.

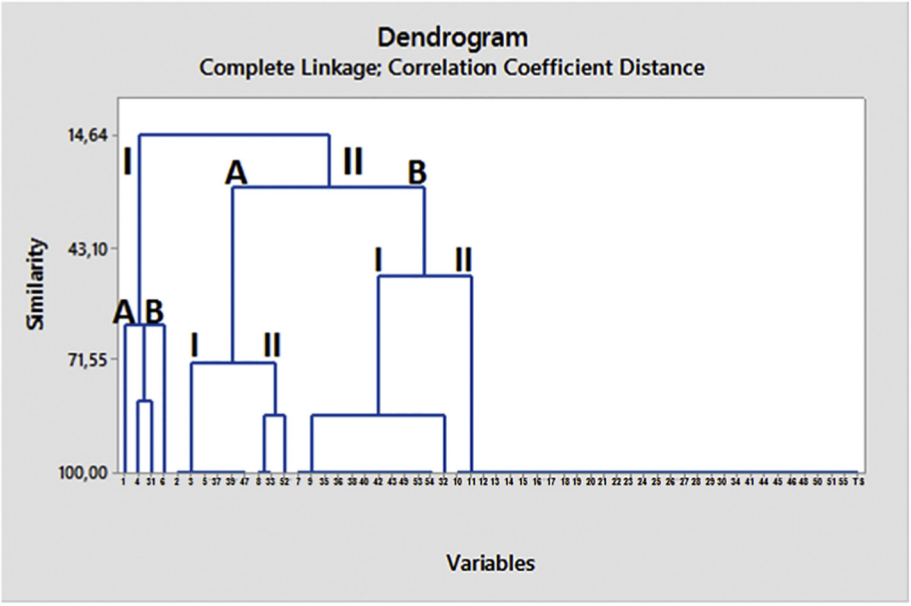

Fig. 3. Similarity analysis of the profiles produced by VNTR1 cluster analysis. 1–55, *Mycoplasma cynos* isolates; TS, Type strain.

depending on which of the three methods is used. These methods have both advantages and disadvantages. RAPD has been used as an easily performed and a highly discriminatory test to type numerous *Mycoplasma* spp. (Rawadi, 1998). On the other hand, RAPD lacks reproducibility, especially when compared to newer techniques (Stakenborg et al., 2006; Van Looveren et al., 1999). RAPD-PCR can be difficult to standardize, and interlaboratory comparison is difficult because the banding patterns often differ from one laboratory to another due to slight differences in technique or use of different thermal cyclers (Marois et al., 2001). It has been reported that use of a single primer leads to comparatively lower sensitivity and specificity, as well as lower detection limits of RAPD-PCR (Sudan et al., 2017). In RAPD-PCR, use of primers only 10 bp long and with high GC content may cause difficulty genotyping bacteria with low GC. This may explain why it is difficult to genotype *M. cynos* by this method.

It was reported that TP-RAPD-PCR tends to amplify a higher number of smaller fragments than the standard RAPD technique, presumably due to inclusion of two primers. TP-RAPD-PCR fingerprinting is thus rapid, sensitive, reliable, highly reproducible, interlaboratory comparable results compliance in results and suitable for experiments with a large number of microorganisms. It can be applied to bacterial taxonomy, ecological studies, and the detection of new bacterial species (Rivas et al., 2001). This procedure avoids several of the problems of PCR fingerprinting that have been reported to date (Hu et al., 1995).

The main advantage of MLVA is in its good reproducibility with high discriminatory power and the ease of performance and interpretation (Kruy et al., 2011). Also it is inexpensive and can be performed using the same equipment used for PCR amplification and agarose gel electrophoresis and is less labour intensive than PFGE (Neoh et al., 2017). The main disadvantage of this technique is that each new assay requires a new optimization, which may be rather time consuming (Vander Horn et al., 1997).

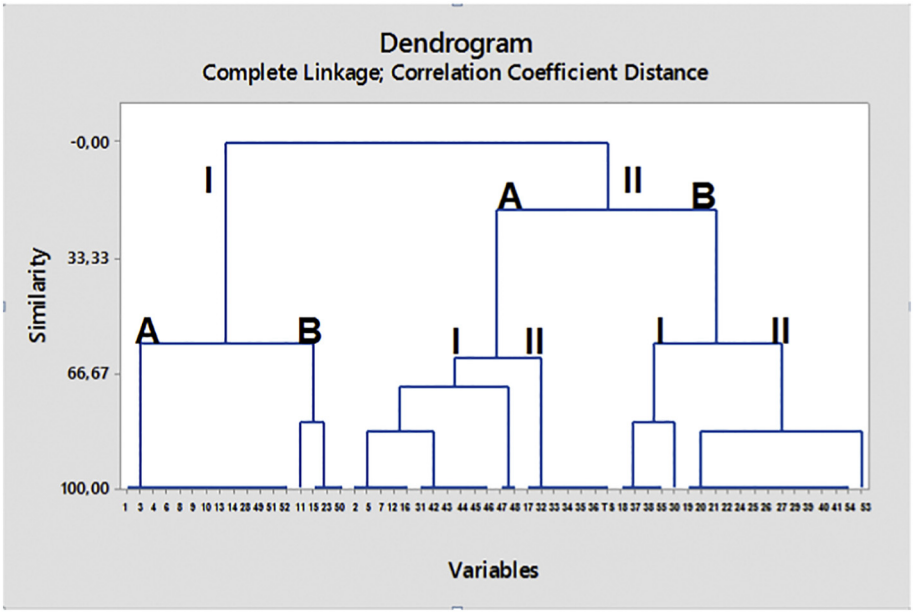

Fig. 4. Similarity analysis of the profiles produced by VNTR1 cluster analysis. 1–55, *Mycoplasma cynos* isolates; TS, Type strain.

This is the first report of *M. cynos* genotyping in dogs using the TP-RAPD-PCR and MLVA methods, and provides foundational data for further studies. All typable isolates were classified into two main groups. The selectivity of VNTR1 is less than the other methods due to the high number of isolates that clustered in the same group. It was detected that the UPGMA values of VNTR3 and TP-RAPD-PCR were similar. Because VNTR3 is located in the essential housekeeping *rpo*B gene of *M. cynos*, this tandem repeat is constrained to only vary such that the reading frame of the gene is retained. It may cause conflict with previous studies which are used to infer phylogenetically relationships among species and to enable identification of the for Mycoplasmataceae species by the partial nucleotide sequences of the rpoB and the 16S 23S rRNA intergenic transcribed spacer (Volokhov et al., 2012). The genetic similarity between the type strain and field isolates was found to be 83.5–92.3% according to the 3 methods. The clustering patterns of RAPD-PCR and TP-RAPD-PCR were more similiar than MLVA. When changing analysis protocol groups of RAPD-PCR with TP-RAPD-PCR and group of VNTR1 with VNTR3, it was noted that some isolates shifted to another cluster. The low number of pet dog isolates precluded statistical validation of differences in the profile being associated with different sources. In the light of these findings, *M. cynos* probably causes fewer infections in pet dogs because of a better environment.

In our study, 6 isolates (49/55) could not be genotyped using RAPD-PCR (Hotzel et al., 1998; Mannering et al., 2009). We attributed this to the use of the Hum4 primer, which was designed for genotyping *M. bovis* (Hotzel et al., 1998) not *M. cynos*. MLVA and TP-RAPD-PCR could generate profiles in all isolates (55/55). It has also been suggested that variations in DNA concentrations can significantly affect the reproducibility of RAPD fingerprinting (Davin-Regli et al., 1995). *M. cynos*, difficulties may be encountered due to a large number of other types of bacteria that grow on the medium (Hong and Kim, 2012). Therefore, in this study, *M. cynos* was extracted from colonies using a gel extraction kit to increase the yield of DNA (Sakmanoglu et al., 2017).

No previous study has reported the discriminatory power (HGDI) of these methods for distinguishing among reference and field isolates (Mannering et al., 2009). In this study, we calculated the discriminatory ability of RAPD-PCR (0.727), TP-RAPD-PCR (0.84), VNTR1 (0.8), and VNTR3 (0.757) for genotyping *M. cynos* isolates. Compared to UPGMA values, TP-RAPD-PCR results were higher than others. Thus, the HGDI, and UPGMA values for these methods in our study were very satisfactory.

In conclusion, the results of this study demonstrate that the TP-RAPD-PCR and MLVA methods are more suitable for studies of high genetic diversity species among populations. These two techniques are more sensitive for detecting the genetic differences in *M. cynos* isolates.

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