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Genetic Diversity of *Pasteurella dagmatis* as Assessed by Analysis of the 16S rRNA and *rpoB* Gene Sequences

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Abstract A total of 16 *Pasteurella dagmatis* strains, including 11 feline and 4 canine isolates as well as one strain isolated from a tiger, were analyzed using partial 16S rRNA and *rpoB* gene sequence comparison. Phylogenetic studies based on both genes revealed that the population of *P. dagmatis* recovered from cats in Poland differs markedly from canine strains, constituting a well-separated cluster within *Pasteurella* sensu stricto species group. The isolate from a tiger seems to represent yet another evolutionary lineage within *P. dagmatis*.

Introduction

Species belonging to the genus *Pasteurella* colonize mainly the upper respiratory tract and the oropharynx of animals, including cats and dogs [4]. Besides their potential pathogenicity to these hosts, they may behave as opportunistic pathogens to humans, causing bite wound infections, complications of underlying respiratory disorders, and systemic infections. Among this group, *Pasteurella multocida* is most frequently reported, although other species (*P. canis*, *P. stomatis*, and *P. dagmatis*) may also be involved [12]. In the medical literature there is a range of reports describing human infections caused by *P. dagmatis*. Most of these conditions have resulted from cat and dog bites [11, 23]. Other infections caused by *P. dagmatis* include peritonitis [3, 24], respiratory tract infection [1], and endocarditis [20].

Pasteurella dagmatis displays some phenotypic similarity (e.g., positive test for urease) to *P. pneumotropica* and Bisgaard Taxon 46, so that these three taxa might be easily misidentified [6, 11]. *P. pneumotropica* is associated predominantly with rodents [4], while bacteria belonging to Bisgaard Taxon 46 were isolated from leopard bite wounds in humans [6].

Accurate identification of the implicated pathogen is of great concern from an epidemiological point of view. In the case of pasteurellae, the phenotypic identification is quite cumbersome, even when automated identification systems are used [7, 11]. For this reason, many laboratories employ molecular methods for identification of *Pasteurella* species, especially sequence analysis of the 16S rRNA gene [16, 24]. Nonetheless, a prerequisite for reliable use of this method is a proven and comprehensive database which comprises a sufficient number of DNA sequences. As an example, it was shown, that strains of *Pasteurella multocida* originating from different animal species may differ genetically, even with regard to a highly conservative 16S rRNA gene [8]. A similar observation was made in the case of *P. dagmatis* [22].

DNA sequence comparison of housekeeping genes has proven to be another valuable tool for phylogenetic

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investigation and identification of different bacteria, including *Pasteurellaceae* [5]. It was found that the *rpoB* gene (encoding the ß-subunit of RNA polymerase) may have a higher discriminatory power than 16S rRNA sequences [17], constituting a reliable complement to the 16S rRNA phylogeny [13].

The objective of this study was to undertake a comparative sequence analysis of the 16S rRNA and *rpoB* genes of *P. dagmatis* (isolates of different host origin), to assess their phylogenetic relationship to *Pasteurella sensu stricto*, *P. pneumotropica*, and Bisgaard Taxon 46 as well as finding out molecular methods for differentiation of various *P. dagmatis* subpopulations.

Materials and Methods

Bacterial Strains and Growth Conditions

The study was performed on 15 field isolates, phenotypically identified as *P. dagmatis*, and the reference strain of this species (*P. dagmatis* CCUG 32658) obtained from the Culture Collection, University of Göteborg, Sweden. Most of the field *P. dagmatis* isolates originated from both diseased and healthy cats and dogs living in the Wrocław and Poznań areas of southwest Poland. Among them, eleven strains were isolated from cats and three from dogs. One strain was isolated from the oral cavity of a tiger kept in the Wrocław Zoo. All data concerning strains used are listed in Table 1. All isolates were grown on 5% sheep blood trypticase soy agar under aerobic conditions and subsequently identified phenotypically and genotypically.

Phenotypic Examination

Phenotypic identification included Gram-staining, catalase, oxidase, production of urease (in Christensen's Medium supplemented with liver digest and glucose [10], production of indole (in Tryptophane Broth [Difco Laboratories, Detroit, MI] with subsequent addition of Ehrlich's reagent), ornithine decarboxylase (using diagnostic tablets ODC, Rosco Diagnostica, Taastrup, Denmark), and production of acid from the following carbohydrates: glucose, sucrose, mannose, maltose, mannitol, sorbitol, and trehalose (in CTA Medium [Becton–Dickinson, Le Pont de Claix, France], supplemented with 1% of appropriate sugar). Results were observed for up to 3 days.

Extraction of DNA

After an overnight cultivation on blood agar, bacterial DNA was extracted using Genomic Mini (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instruction.

Amplification and Partial Sequencing of the 16S rRNA and *rpoB* Genes

For amplification of 1403- and 560-bp fragments of the 16S rRNA and *rpoB* genes, respectively, previously described primers [13, 14] were used. The reaction mixture

Table 1 Bacterial strains used in this study

Strain designation[a]	Origin	Time and place of isolation
P. dagmatis strain 6/4	Cat, throat	Mar 2005/P
P. dagmatis strain 13/5	Cat, throat	Apr 2005/P
P. dagmatis strain 15/1	Cat, throat	Apr 2005/P
P. dagmatis strain 95	Cat, oral cavity	Jun 2004/W
P. dagmatis strain 167/2	Cat, *rhinitis*	Nov 2006/W
P. dagmatis strain 182/1	Cat, *conjunctivitis*	May 2007/W
P. dagmatis strain 185/2	Cat, *rhinitis*	Jul 2007/W
P. dagmatis strain 223/2	Cat, throat	Jun 2009/W
P. dagmatis strain 243	Cat, *stomatitis*	Apr 2010/W
P. dagmatis strain 246/1	Cat, *rhinitis*	Jun 2010/W
P. dagmatis strain 247	Cat, *rhinitis*	Jun 2010/W
P. dagmatis strain 123/2	Dog, throat	Mar 2005/W
P. dagmatis strain 149/1	Dog, throat	Feb 2006/W
P. dagmatis strain 505	Dog, throat	Oct 2008/W
P. dagmatis strain 197	Tiger, oral cavity	May 2008/W
P. dagmatis CCUG32658	Dog, throat	

[a] The GenBank accession numbers for new sequences are as follows: 16S rRNA gene of *P. dagmatis* strain 197: HQ538835; 16S rRNA gene of *P. dagmatis* strain 247: HQ538836; 16S rRNA gene of *P. dagmatis* strain 243: HQ538837; *rpoB* gene of *P. dagmatis* strain 247: HQ538838; *rpoB* gene of *P. dagmatis* strain 197: HQ538839

P the Poznań area, *W* the Wrocław area

(25 µl) contained 10 mmol/l Tris–HCl, pH 8.8, 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 0.08% Nonidet P40 (Fermentas, Vilnius, Lithuania), 5 pmol of each primer (Institute of Biochemistry and Biophysics, Warsaw, Poland), 0.2 mmol/l of each deoxyribonucleotide (Fermentas), 2 U of Taq DNA polymerase (Fermentas), and 2 µl of DNA. Forty PCR cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 120 s were performed. PCR products were purified (by adding 10 U of *E. coli*-Exonuclease I and 2 U of Shrimp Alkaline Phosphatase [Fermentas] to 5 µl of the reaction mixture) and sequenced—on both DNA strands—using the DYEnamic ET terminator cycle sequencing kit ABI Prism™ (Amersham Biosciences Europe GmbH, Germany).

Molecular Identification and Analysis of Nucleotide Sequences

Searches for homologous DNA sequences in GenBank were performed using the BLAST algorithm [2]. All sequences were subjected to multiple alignment using ClustalW program (http://www.mbio.ncsu.edu/bioedit/bioedit.html). For comparative studies, a 1308-bp fragment of the 16S rRNA gene and 501-bp fragment of the *rpoB* were selected (corresponding to positions 83–1390 of the *E. coli* 16S rRNA sequence, accession number J01859, and positions 1543–2043 of the *E. coli rpoB* gene, accession number AB488804, respectively). Phylogenetic analyses were performed using the MEGA version 3.1 software [15]. Dendrograms were generated by the neighbor-joining method, with bootstrap analysis (corresponding to 500 replications), included to assign confidence values.

Digestion of PCR Products using Restriction Endonuclease

A restriction map of 1403-bp 16S rRNA sequences, obtained from feline, canine, and tigrine *P. dagmatis* isolates, was constructed using BioEdit Software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). For digestion, 476-bp fragments of this gene containing polymorphic site for *Taq*I restriction enzyme were selected (the following oligonucleotide primers were used: for, 5′-CGAACGG TAGCAGGAAGAAAGCTTG-3′; rev, 5′-GWATTACCG CGGCKGCTG-3′). Thirty-five PCR cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s were performed. The PCR products were subsequently digested in a mixture containing 1 µl (10 U) of *Taq*I restriction enzyme (Fermentas), 3 µl of 10× *Taq*I Buffer, 10 µl of PCR mixture, and 16 µl of water. Reaction was performed at 65°C for 60 min, and 15 µl of digestion mixture was resolved in 2.5% agarose gel stained with ethidium bromide.

Results

Phenotypic Identification

All isolates identified phenotypically as *P. dagmatis* were catalase-, oxidase-, indole-, and urease-positive. The isolates produced acid from glucose, sucrose, maltose, mannose (the feline isolate 6/4 was negative), and trehalose (with the exception of the reference strain CCUG 32658). Two strains (the feline isolate 6/4 and the strain from a tiger) also produced acid from sorbitol, and one canine isolate (149/1)—from mannitol. All strains (except for the isolate 505 recovered from a dog) were negative for ornithine decarboxylase.

Molecular Identification and Phylogenetic Study

Analysis of the 16S rRNA nucleotide sequences using the BLAST algorithm revealed that canine isolates have a high degree of sequence similarity (99.78–99.85%) with the *P. dagmatis* type strain AY362920. In contrast, all feline isolates were practically identical (99.47–99.85%) to the *P. pneumotropica* NCTC 10827 strain (accession number in GenBank AF224296). AF224296 was also the highest scoring sequence for the strain isolated from a tiger. In this case, the similarity was lower and amounted to 98.58%.

16S rRNA sequences of feline isolates were highly homologous, with the only polymorphic nucleotide site (A/T substitution) located in position 842 (relative to AF224296). It was found that feline *P. dagmatis* sequences display several unique nucleotide sites which distinguish it from canine *P. dagmatis* strains and distinguish it from the isolate recovered from a tiger (Table 2).

A phylogenetic analysis of 16S rRNA sequences was performed with 36 sequences, including 16 isolates determined in the present study and 20 sequences deposited in GenBank (14 sequences of *Pasteurella* sensu stricto core group, four of *P. pneumotropica*, and two of Bisgaard Taxon 46). An evolutionary tree constructed by the neighbor-joining method was shown on Fig. 1. Of the 19 nodes, 15 were also supported by bootstrap values higher than 50%. Analyzed sequences were divided into nine clusters. Cluster IA was composed of the *P. pneumotropica* strain NCTC10827, two strains recovered from cats, designated as *P. dagmatis*-like 5/8 and 8/4 (sequences retrieved from GenBank), and 11 feline *P. dagmatis* isolates analyzed in this study. Members of this group displayed only a 97.48–97.60% sequence similarity with the *P. dagmatis* type strain (AY362920). Cluster IB contained the strain 197 from a tiger and the feline *P. dagmatis*-like strain 1/1 (from GenBank). *Pasteurella stomatis* and *P. canis* constituted clusters II and III, respectively. Four canine *P. dagmatis* isolates, characterized by the present

Table 2 Distribution of polymorphic nucleotide sites among 16S rRNA sequences of *P. dagmatis* of different host origin

	Nucleotide positions (relative to the sequence of *P. dagmatis* strain CCUG12397^T [AY362920])							
	157–159	162–165	183–186	448–449	589–591	599–601	980–984	991–995
Canine *P. dagmatis* ($n = 4$, incl. the reference strain CCUG 32658)	GTA	GAGA	TTCG	AT	CGA	TTG	GCCGT	ATGGC
Feline *P. dagmatis* ($n = 11$)	T..	.TA.	A.TT	G.	T.G	C.A	AA.TC	GA.TT
Pasteurella dagmatis strain from a tiger ($n = 1$)	A.C	TGAG	..A.	GC	T.G	C.A	AA.TC	GA.TT

The dots represent sites where the nucleotides match those of the topmost sequence

authors, as well as the type strain of this species (CCUG12397^T, sequence obtained from GenBank), fell into cluster IV. Clusters V, VI, and VII were composed of Bisgaard Taxon 46, *P. multocida* subsp. *septica*, and *P. multocida* subsp. *multocida/P. multocida* subsp. *gallicida* complex, respectively. Three sequences of *P. pneumotropica* (other than AF224296) were grouped in separate clusters VIII and IX with low similarity to all other sequences of the *Pasteurella* sensu stricto core group.

Comparison of *rpoB* sequences confirmed the genetic distinctness of the feline subpopulation of *P. dagmatis*. All the isolates recovered from cats had the same nucleotide sequence of the *rpoB* gene that displayed a 97.4% similarity (12 mismatches on a total of 501 nucleotides compared) with the *P. dagmatis* type strain CCUG12397^T (AY362966). On the other hand, sequences of the canine isolates showed a 98.6–99.6% similarity (2–7 mismatches out of 501 nucleotides) to AY362966. The isolate from a tiger was most distantly related and had only a 94.4% similarity with *P. dagmatis* CCUG12397^T (28 mismatches).

The *rpoB* gene tree (Fig. 2) was strongly supported by high bootstrap values (higher than 80% accounted for 12 out of 17 nodes). In this dendrogram, canine and feline isolates of *P. dagmatis* also clustered in separated groups. However, unlike the phylogeny derived from the 16S rRNA gene, an analysis of the *rpoB* sequences revealed that both canine and feline subpopulations of this species are more closely related to each other than to other members of the *Pasteurella* sensu stricto core group. The isolate from a tiger constituted a distinct *P. dagmatis*-like taxon, less closely related to the above *P. dagmatis* subgroups. The remaining *rpoB* gene sequences analyzed in this study were grouped in separate taxon-specific clusters. In both 16S rRNA and *rpoB* evolutionary trees, strains of *P. pneumotropica* (other than NCTC10827) constituted a group clearly different from feline isolates of *P. dagmatis*.

Restriction Fragment Length Polymorphism of the16S rRNA Gene

The restriction nuclease *Taq*I was shown to cleave 476-bp fragments of the 16S rRNA gene from canine *P. dagmatis* isolates, giving products of 125 and 351 bp. Amplicons obtained from DNA of feline isolates (and the strain from a tiger) remained undigested (Fig. 3).

Discussion

Comparison of 16S rRNA and *rpoB* gene sequences reveals that *P. dagmatis* is a heterogeneous species, containing at least two host-specific lineages. In this study, it was found that canine and feline isolates of *P. dagmatis*, recovered from animals living in Poland, differ genetically from one another. In addition, one strain from a tiger was found that seems to represent yet another taxon related to *P. dagmatis*. Key biochemical characteristics are very similar among particular groups of this species. This means that they are indistinguishable under routine phenotypic identification.

Although *P. dagmatis* has been isolated from different domestic animals [4, 9] as well as from human clinical specimens [23, 24], the genetic diversity of the species has not been explored extensively so far. Recently, atypical *P. dagmatis* isolates recovered from cats were described [22]. While the basic phenotypic properties of these strains were characteristic of *P. dagmatis*, they were referred to as "*P. dagmatis*-like" due to unusual colony morphology. Sequencing of the 16S rRNA gene of three selected isolates showed only a 97% similarity with known sequences of *P. dagmatis*; instead, they were strikingly similar to the reference strain *P. pneumotropica* NCTC10827 (GenBank accession AF224296). The results of the present investigation revealed that all feline *P. dagmatis* strains constitute a coherent group displaying almost 100% similarity of the 16S rRNA gene with sequences of Hungarian *P. dagmatis*-like strains 5/8 and 8/4 (GU177868 and GU177869) and with the sequence of *P. pneumotropica* NCTC10827. In contrast, all canine *P. dagmatis* isolates had the same 16S rRNA sequence that is consistent with sequences of *P. dagmatis* from GenBank. This might be of phylogenetic and epidemiologic significance, as it indicates that strains of *P. dagmatis* or *P. dagmatis*-like, isolated from cats (at least in Hungary and Poland) constitute a monophyletic group, differing from canine isolates. Whether or not this

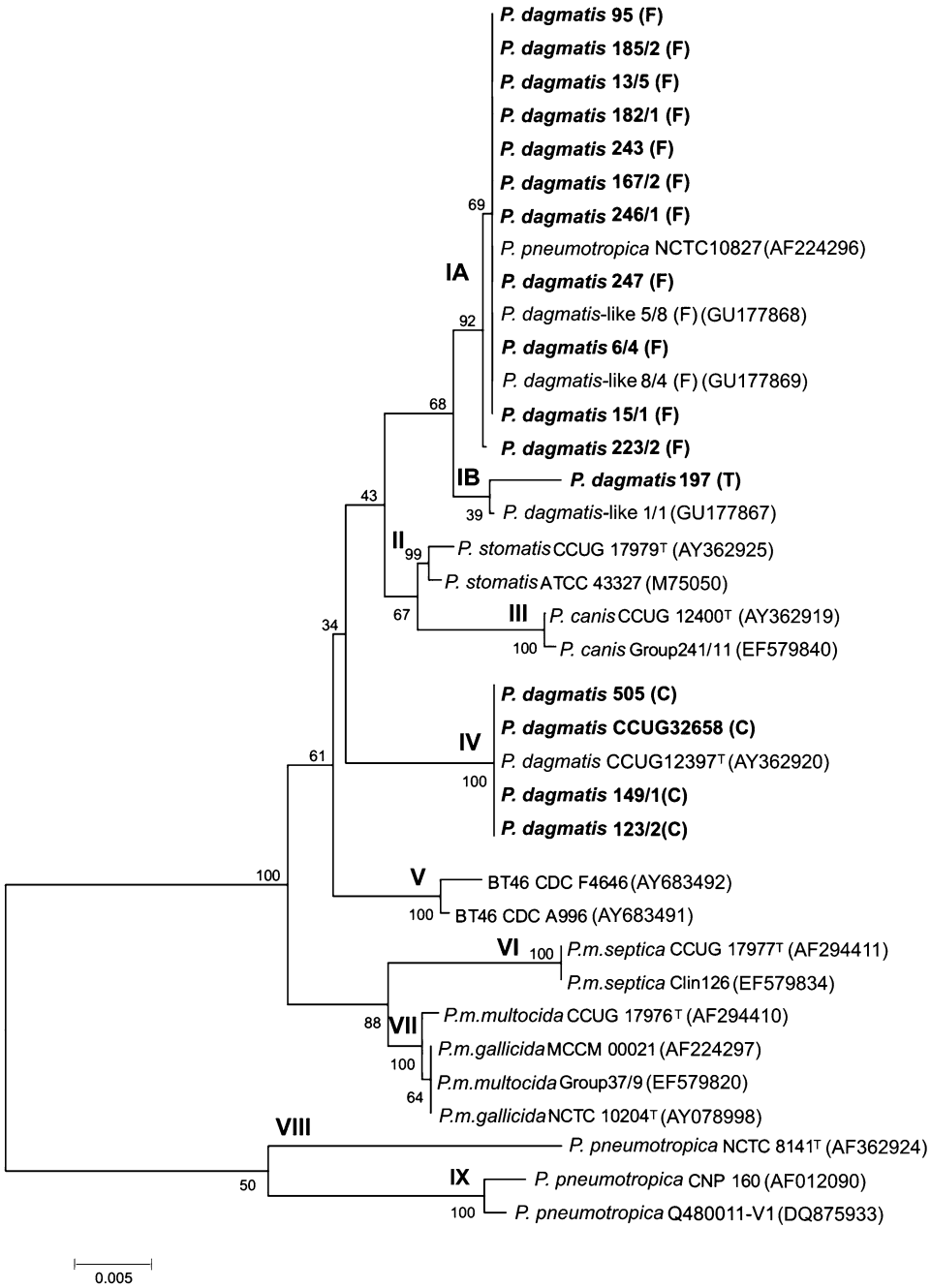

Fig. 1 Phylogenetic tree of 36 *Pasteurella* strains based on 16S rRNA sequences (1308 bp fragments). The tree was constructed using the neighbor-joining method. Bootstrap values of 500 replications are indicated as percent confidence values for particular branching. Sequences indicated in *bold* were determined in this study. *CCUG* Culture Collection, University of Göteborg, Sweden; *NCTC* National Collection of Type Cultures, UK; *MCCM* Medical Culture Collection Marburg, Germany; *ATCC* American Type Culture Collection, USA; *CNP* Centre National des Pasteurella, France; *CDC* Centers for Disease Control and Prevention, USA; ^{T} type strain; (*F*) isolates from cats; (*C*) isolates from dogs; (*T*) the isolate from a tiger

phenomenon is unique to Central Europe remains to be elucidated. Some evidence indicates, however, that feline-specific *P. dagmatis* strains have also been isolated in Western Europe. As an example, a case of peritonitis in a human caused by *P. dagmatis* was reported [24]. According to a case history, cats were suspected of being the source of infection. The 16S rRNA gene of the isolated strain was sequenced, giving only 97% similarity with sequence of *P. dagmatis* from GenBank.

The results of the investigation indicate that *P. pneumotropica* NCTC10827 may have been misclassified and its 16S rRNA gene sequence incorrectly labeled. Feline isolates that were identified phenotypically as *P. dagmatis* (both Hungarian [22] and those tested in this study), have over a 99.4% similarity of the 16S rRNA gene with the above strain, being identified as *P. pneumotropica* using the BLAST algorithm. *P. pneumotropica* is encountered predominantly in rodents yet its biovar Heyl was also isolated from a cat [4]. This species is positive for ornithine decarboxylase [11, 21], while all the feline isolates were ODC negative. Similarly, negative ODC-reaction was found in Hungarian *P. dagmatis*-like strains as well as in

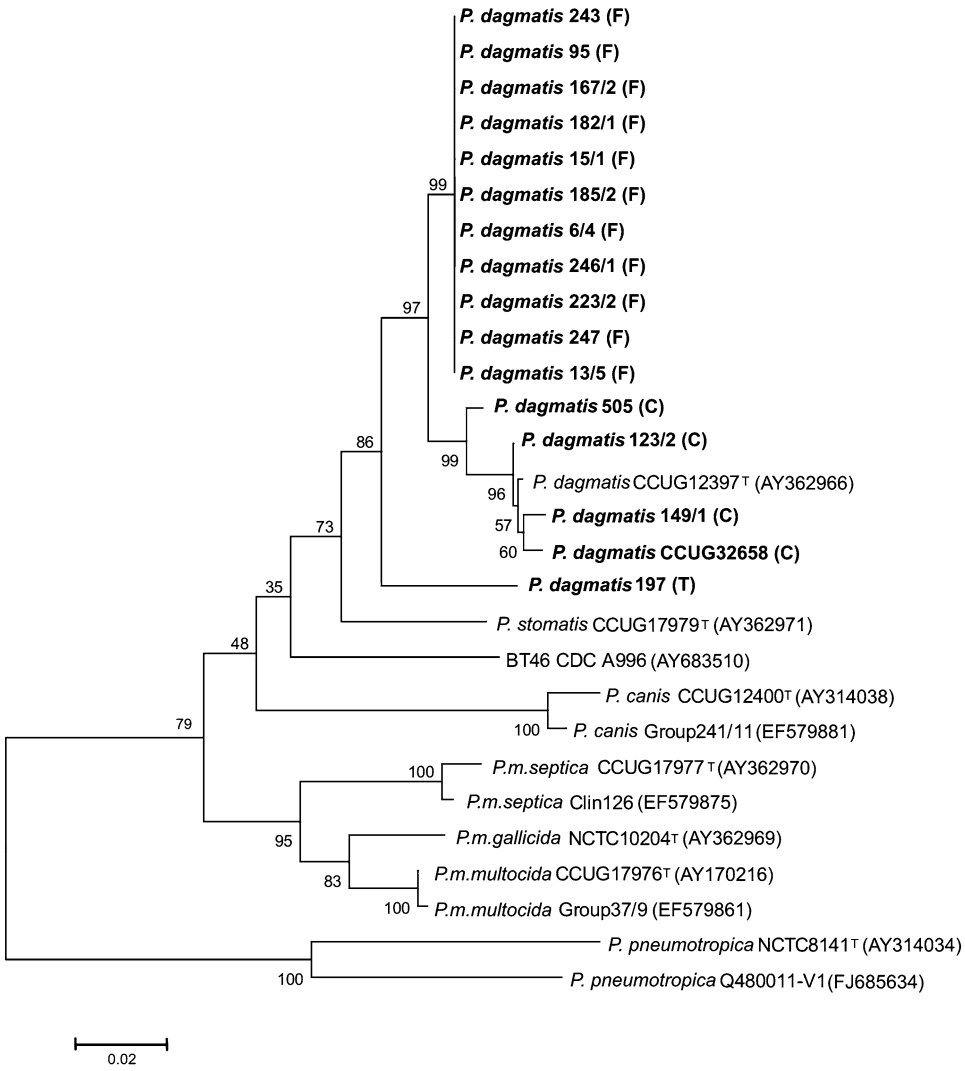

Fig. 2 Phylogenetic tree of 28 *Pasteurella* strains based on *rpoB* sequences (501 bp fragments). The tree was constructed using the neighbor-joining method. Bootstrap values of 500 replications are indicated as percent confidence values for particular branching. Sequences indicated in *bold* were determined in this study. *CCUG* Culture Collection, University of Göteborg, Sweden; *NCTC* National Collection of Type Cultures, UK; *CDC* Centers for Disease Control and Prevention, USA; ^{T} type strain; (*F*) isolates from cats; (*C*) isolates from dogs; (*T*) the isolate from a tiger

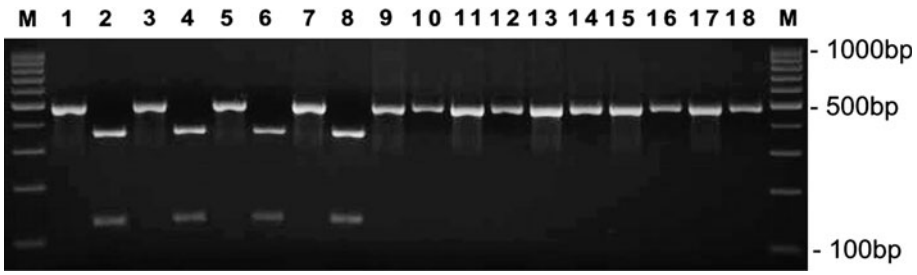

Fig. 3 Agarose gel electrophoresis of a 476-bp fragment of the *P. dagmatis* 16S rRNA gene. *Lanes 1–8* represent amplicons obtained from DNA of canine isolates, *lanes 9–16* from DNA of feline isolates, and *lanes 17–18* from DNA of the isolate from a tiger. For each isolate, intact amplicons (*lanes* with *odd numbers*) and *Taq*I-digested ones (*lanes* with *even numbers*) were run. *M* size marker (GeneRuler™ 100 bp DNA Ladder [Fermentas])

P. pneumotropica NCTC10827 [22]. NCTC10827 was reported to be an atypical strain, even within heterogeneous *P. pneumotropica* species [18, 21]. The similarity of the NCTC10827 16S rRNA gene with other *P. pneumotropica* sequences (deposited in GenBank) is relatively low, reaching at most 94%. Analysis of the *sodA* gene also confirms the genetic distinctness of the NCTC 10827 strain from other *P. pneumotropica* isolates [22], indicating that it should be placed within the *Pasteurella* sensu stricto core group. The strain NCTC10827 was isolated from human blood and identified based on its biochemical properties (mainly a positive urease reaction) [19]. The ability to

produce gas from some carbohydrates, reported by Rogers et al. [19], could also argue that this strain belong to the *P. dagmatis*-like group rather than to *P. pneumotropica*.

In both phylogenetic trees based on the 16S rRNA and *rpoB* genes, all feline isolates investigated here constitute a well-separated cluster within the *Pasteurella* sensu stricto core group. Described genetic differentiation of *P. dagmatis* may be of some importance in epidemiologic studies, especially in cases of *P. dagmatis*-infections of unknown origin in humans. Feline and canine isolates were distinguishable using the restriction enzyme *Taq*I, which could be used for laboratory diagnostics.

It seems that the strain 197, isolated from a tiger, constitutes another evolutionary lineage within *P. dagmatis*. Based on 16S rRNA nucleotide composition, it has the highest level of similarity with the *P. dagmatis*-like isolate 1/1 GU177867 (99.08%) and *P. pneumotropica* NCTC10827 (98.58%). The isolate from a tiger differed phenotypically from Taxon 46 in positive sucrose fermentation. In addition, results of phylogenetic analyses based on both 16S rRNA and *rpoB*-trees indicate that the isolate from a tiger is more closely related to *P. dagmatis* than to Bisgaard Taxon 46.

In summary, it seems that there are several subpopulations within *P. dagmatis*, associated with specific hosts. Further studies on this organism which take into consideration isolates of various geographical distribution and host range should be performed. It would be interesting not only for phylogenetic analyses but also from epidemiological point of view, as *P. dagmatis* and *P. dagmatis*-like bacteria are involved in a number of bite wounds and other infections in humans.

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