The 16S-23S rDNA internal transcribed spacer (ITS) sequences for analysis of the phylogenetic relationships among species of the genus *Fusobacterium*

Georg Conrads,[1,2] Marina C. Claros, [3] Diane M. Citron,[1] Kerin L. Tyrrell, [1] Vreni Merriam [1], and Ellie J.C. Goldstein [1]

[1] R.M. Alden Research Laboratory, Santa Monica-University of California at Los Angeles Medical Center (UCLA), Santa Monica 90404, California

[2] Institut für Medizinische Mikrobiologie, Universitätsklinikum (RWTH), D-52057 Aachen, Germany

[3] Institut für Medizinische Mikrobiologie und Infektionsepidemiologie, Universität Leipzig, D-04103 Leipzig, Germany

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Corresponding author:

Georg Conrads, Dr.rer.nat.
R.M. Alden Research Laboratory,
Santa Monica-UCLA Medical Center
2021 Santa Monica Blvd., Suite 740E
Santa Monica
CA 90404.
Phone (310) 319-4325
Fax: (310) 315-3662
E.mail: gconradsphd@aol.com

SUMMARY

The 16S-23S rDNA internal transcribed spacer (ITS) regions of all currently defined *Fusobacterium* species, and related taxons such as *Leptotrichia buccalis, Sebaldella termitidis* and *Streptobacillus moniliformans*, were analysed to examine inter- and intra-species as well as subspecies relationships. For the ITS-amplification, a new eubacterial universal primer pair was designed and used. The majority of the *Fusobacterium* strains along with *Leptotrichia buccalis* showed one major and 2-3 weaker, distinct bands (short and long versions) with a length between 800-830 bp and 1000-1100 bp. Nevertheless, six other patterns were also found within the genus *Fusobacterium*, demonstrating its heterogeneity. The ITS region was sequenced and found to consist both of conserved motifs, which functioned as a framework for alignment, and of variable sites which provided high phylogenetic resolution. Analyses of the ITS-DNA sequences and ITS relative length (short version) allowed species and subspecies differentiation in most cases. The results confirmed the strikingly distant relationship of *Fusobacterium prausnitzii* to the genus *Fusobacterium*. *F. nucleatum* subspecies along with *F. naviforme, F. simiae,* and *F. periodonticum* formed a cluster with an inherent high potential for diversification. Other clusters were formed by *F. necrophorum* subspecies with *F. gonidaformans* and by *F. varium* with *F. mortiferum* and *F. ulcerans*. *F. russii* as well as *F. perfoetens* formed separate branches. *F. necrophorum* subspecies *necrophorum* and *funduliforme* on the one hand, and *F. varium* and *F. mortiferum* on the other, were found to be very similar, even at the high resolution ITS level.

INTRODUCTION

Fusobacteria are obligately anaerobic non-spore-forming gram-negative, nonmotile, pleomorphically rod-shaped bacilli. The DNA base composition within this genus is heterogeneous. Whereas the majority of strains have a restricted range of 26 to 34 mol% G+C, strains of *F. prausnitzii* (ATCC 27768^T and 27766: 49 to 57 mol%) and *F. naviforme* (ATCC 25832: 49 mol%) are well outside this range. It was therefore suggested that they may belong to a different taxonomical group (Bennett & Eley, 1993; Gharbia & Shah, 1990; Wang *et al.*, 1996).

It is widely accepted that comparative analysis of small-subunit rRNA sequences is a powerful tool for investigating the phylogenetic relationships of especially biochemically inert micro-organisms. Nevertheless, because of the limited resolution of 16S sequences, different data mining approaches can lead to different results, as has been reported for *F. alocis*. The 16S rRNA sequence-based distance matrix used by Lawson *et al.* (1991), confirmed the similarity between *F. alocis* and the *F. nucleatum*-group, whereas the recent publication by Jalava & Eerola (1999), led to the reclassification of this species as the gram-positive *Filifactor alocis*.

Sequence polymorphism and lengths found in the 16S-23S rDNA internal transcribed spacer (ITS) is increasingly being used as a tool for differentiation of bacterial species and subspecies (Guasp *et al.*, 2000; Motoyama & Ogata, 2000). The higher number of variable sites typical for the ITS sequence (Soller *et al.*, 2000) seem to overcome some of the apparent limitations of the phylogenetic resolution of the 16S rDNA. Although variable, the spacer is sufficiently conserved to guarantee a stable classification (Anton *et al.*, 1998; Gurtler 1999; Iteman *et al.*, 2000; Perez Luz *et al.*, 1998).

This study was performed to differentiate and tree strains of the genus *Fusobacterium* and other related members of the “fusobacterial group”. In order to amplify the 16S-23S ribosomal ITS, a new eubacterial PCR primer set was designed. The length and pattern of the amplificons together with patterns of DNA sequence variability were used to clarify the phylogenetic relationship of *Fusobacterium* subsp. and related genera.

METHODS

Bacterial strains, culture conditions and DNA extraction. The bacterial strains used in the present study are listed in **Table 1** and were either directly received from a type culture collection or phenotypically characterized at the R.M. Alden Research Laboratory according to the original Bergey's Manual description. Fusobacterial strains and *Leptotrichia buccalis* were cultivated at 37°C on Brucella agar (Anaerobe Systems) under anaerobic conditions using an anaerobic chamber; aerobes (used for contrast) were cultivated on blood agar plates (Hardy Diagnostics). DNA was extracted using the DNeasy Tissue Kit (QIAGEN Inc.).

PCR amplification and DNA sequence analysis. The 16S primer SPF (5' GGT GTG ACG GGC GGT GTG TAC 3', *E.coli* position 1391-1411) was designed on the basis of universal 16S sequence information (RDP, Ribosomal Database Project II, Il, USA),. The target sequence of the degenerated 23S primer SPR [5' GGT (TG)CT TTT C(GA)C CTT TCC 3`, *E. coli* position 468-485] was highly conserved among eubacterial large subunit sequences (RDP). PCR was carried out using a Biometra Uno I (Biometra) thermocycler in a volume of 100 µl containing 1 X PCR buffer, 1.5 mM $MgCl_2$, 2 units Taq-polymerase (Boehringer Mannheim), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), 10 pmol SPF forward primer, 100 pmol reversed primer SPR**,** and 100 ng of template nucleic acids. Primer oligonucleotides were synthesised using a DNA synthesiser (OLIGO 1000, Beckman). The amplification was performed using the following temperature profile and 30 cycles: denaturation - 1 min at 94°C**;** annealing - 1 min at 50°C**;** elongation - 2.5 min at 72°C.

Amplification products (aliquots of 10 µl) were separated electrophoretically on a 2% (w/v) macro agarose gel in 1x TPE (80 mM Tris-phosphate, 2 mM EDTA, pH 7.5) for a minimum of 18h at 30V.

After purification using the Wizard DNA Clean-up system (Promega), the spacer DNA was directly sequenced in duplicate using a Taq Dye-Deoxy terminator cycle sequencing kit (Applied

Biosystems) and an automatic capillary DNA sequencer (API PRISM 310; Applied Biosystems). Sequences were assembled using the program Vector NTI Suite (InforMax) and aligned using the program GeneDoc (Nicholas & Nicholas, 1997). A phylogenetic tree was constructed by the neighbour-joining method and the program Clustal W (Jeanmougin *et al.*, 1998) using *Fusobacterium prausnitzii* as an outgroup.

RESULTS

Approximations of ITS lengths were obtained from agarose gels, as demonstrated in **Fig. 1**. **Table 1** gives the number and lengths of different amplificons found in each species and strain tested. In some species (e.g. *F. mortiferum*, *F. ulcerans*, or *F. gonidioformans*) larger amplificons, most likely ITS dimers of 1900 to 2200 bp length, occurred facultatively in addition to the monomeric ITS (800 to 1080 bp).

According to this, only a few species, like *F. nucleatum* subsp. *polymorphum*, *F. periodonticum*, and *F. prausnitzii*, showed a unique pattern of PCR bands in gel electrophoresis. In addition, *F. nucleatum* subsp. *animalis* and *F. necrogenes* could be identified either by small differences in the lengths of amplificons or by the comparative intensities of their bands. In general, however, it was not possible to differentiate fusobacterial species by comparing ITS-gel-electrophoretical profiles alone. Further discrimination without need of sequencing could be possible by ITS restriction, since we found variations in the following restriction sites: *Eco*RI (bp 676-731), *Hind*III (bp 303-454) and *Ava*I (bp 358-473). *F. prausnitzii* was the only *Fusobacterium* species without an *Eco*RI restriction site. Reference strains of the chosen relatives *Sebaldella termitidis* or *Streptobacillus moniliformans* and the more distantly related species such as *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, *Escherichia coli*, *Eubacterium lentum*, *Porphyromonas gingivalis*, and *Streptococcus mutans*, demonstrated completely different gel-electrophoresis patterns from fusobacteria using the described ITS-directed PCR method (data shown only for *Sebaldella termitidis* and *Streptobacillus moniliformans,* **Table 1.**).

Sequencing the purified amplificons using SPF and SPR as primers was performed in duplicate and led to nearly ambiguity-free sequence determination by comparing both runs and directions. **Fig. 2** shows the sequence deduced from *F. nucleatum* subsp. *nucleatum* ATCC 25586^T with conserved regions highlighted for orientation. A similarity matrix table and an alignment file of DNA-DNA hybidization data for all 33 taxa sequenced is available in IJSEM Online.

In the spacer region approximately 3.6 ambiguities appeared. Species demonstrating ITS patterns either with confluent bands, such as *F. nucleatum* subsp. *polymorphum,* or with 4 bands such as *F. gonidiaformans*, had more unresolvable ambiguities (13 maximum) in the deduced sequence. Since the short ITS version was obviously favoured in the PCR and amplified in much higher numbers compared to the long fragments (which most likely contain t-RNA gene insertions), the resulting sequence represents clearly the t-RNA-free version only. A database search of t-RNA consensus sequences and comparison with our fusobacterial spacer DNA revealed no matches.

The phylogram deduced from the ITS sequences is demonstrated in **Fig. 3.** From one type strain (*F. varium* ATCC 8501^T) two cultures from different sources (strain collection University of Leipzig, Germany and R.M. Alden Research Laboratory, Santa Monica, CA) were sequenced as an internal control and (Seq. 1 and Seq. 2 in **Fig. 3**) found to be >99% identical. The different strains of most *Fusobacterium* species matched on a 96-100% level. Excluding N`s, relevant differences were found only among strains within the *F. nucleatum* subspecies *nucleatum* and *animalis*, respectively. According to the spacer sequences, three major groups could be discriminated.

The species of the *F. mortiferum*/*varium*/*ulcerans*-group were found to be very closely related (93-97% similarity) and related to *F. necrogenes* (79-81%). A second group was formed by all *F. nucleatum* subspecies along with *F. simiae, F. periodonticum* and *F. naviforme* which showed a match between 81 and 95%. A third group was formed by both of the *F. necrophorum* subspecies and *F. gonidiaformans* (79-98%).

F. russii represents an individual evolutionary branch within the fusobacterial main cluster and *F. perfoetens* is distantly related to all other core species with a similarity of only 71-78%.

F. prausnitzii, represented in this study by two reference strains, appeared unrelated to fusobacteria (48% similarity to the type species *F. nucleatum* subsp. *nucleatum*). Representatives of the most closely related genera (by 16S rRNA data), *Leptotrichia* (*L. buccalis* ATCC4201^T), *Sebaldella* (*S. termitidis* ATCC 33386^T), and *Streptobacillus* (*S. moniliformans* 14647^T) showed a closer relationship (66, 66, 63 %) to the fusobacterial type species than did *F. prausnitzii*. Even *E.coli* (sequence imported from strain RIMD 0509952, GenBank accession no. AB035920) demonstrated a higher similarity (50%) to the fusobacterial type species after deletion of the alanine tRNA from the spacer region.

DISCUSSION

Deducing bacterial phylogenetic relationships from 16S-23S rDNA ITS-sequences seems to have several advantages over using 16S rRNA/DNA alone. The phylogenetic differences are not only expressed in the sequence information itself but also by the different lengths of amplificons and, in some cases, the formation of distinct band patterns by gel electrophoresis resulting from variations among the rrn operons in the same strain (Christensen *et al.*, 2000; Iteman *et al*., 2000).

The taxonomy of fusobacterial species and some related genera and species is still a scientific riddle, especially with respect to the five controversial subspecies in *Fusobacterium nucleatum* (Gharbia & Shah, 1992) and the two in *F. necrophorum* (Okwumabua *et al.*, 1996; Shinjo *et al.*, 1991). *F. alocis* and *F. sulci* have already been reclassified as *Filifactor alocis* and *Eubacterium sulci*, based on 16S rDNA sequences (Jalava & Eerola, 1999).

PCR amplification of the ITS region using newly designed primers and 33 fusobacterial strains showed striking differences after gel electrophoresis in only a limited number of species. Nevertheless, patterns consisting of one to a maximum of four bands were produced with each strain tested. Thus, most *Fusobacterium* species exhibit different variants of the 16S-23S rDNA spacer as described for other taxons (Graham *et al.*, 1997; Gurtler *et al.*, 1999; Motoyama & Ogata,

2000). Within a species or subspecies, as we have seen in our testing, the pattern and the deduced sequence is relatively constant and matches on a 97-100% level, with *F. nucleatum* subsp. *nucleatum* being the only exception (<97%).

The high resolution of ITS sequences led to a striking departure of *F. prausnitzii* from the core fusobacterial group in our analysis. This confirms the finding of a previous study that used 16S rDNA sequences to demonstrate that *F. prausnitzii* clusters with *Eubacterium* and *Clostridium* group III and IV (Wang, et al., 1996). Clearly, *F. prausnitzii* must be reclassified.

Because of its 49 mol% G+C contents, it has been suggested that *F. naviforme* may also be outside the fusobacterial group (Gharbia & Shah, 1990). However, the *F. naviforme* original ATCC 25832 strain tested in our study related by 93% sequence similarity to *F. nucleatum* subsp. *nucleatum* ATCC 25586; it showed a typical morphology (boatshaped), a typical biochemical profile (weak glucose fermentation, a positive indole reaction), and was resistant to the 5 µg vancomycin disk. Interestingly enough, "*F. naviforme* strain ATCC 25588" obtained from other laboratories showed *Eubacterium*-like spacer sequences, a different biochemical profile from our strain and was susceptible to vancomycin suggesting a gram-positive organism (sequence data not included in this study). Therefore, we recommend that the identity of *F. naviforme*-strains other than those received directly from the ATCC or RMA collections be confirmed according to the original Bergey's Manual description.

Basically, our ITS data support the validity of subspecies within *F. nucleatum* and its separation from *F. periodonticum*. However, the ITS sequences in this cluster, which includes *F. simiae* and *F. naviforme*, showed a "fan-like" branching. Together with the intra-subspecies diversity at a 96% level in *F. nucleatum* subsp. *nucleatum*, this fusobacterial branch seems to have an increased potential for genetic diversity in general. Further subtyping will undoubtedly occur after additional strains are sequenced, but a re-integration of all or some subspecies should also be considered to limit the number of fusobacterial taxons.

In contrast, ITS-sequencing could barely differentiate between *F. necrophorum* subspecies *necrophorum* and *funduliforme*, or between *F. varium* and *F. mortiferum*, since the spacer sequences of these are up to 98% identical with each other. And yet, *F. varium* and *F. mortiferum* can be differentiated by cell morphology and a few biochemical tests, such as esculin reaction and lactose fermentation (Claros *et al.*, 1999).

The species *F. russii* and *F. perfoetens* formed individual branches, quite apart from the clusters formed by *F. nucleatum* subspecies/ *F. naviforme*/ *F. simiae/ F. periodonticum,* by *F. necrophorum* subspecies/*F. gonidaformans*, and by *F. varium*/*F. mortiferum*/*F. ulcerans*. This might explain some very unique features found in these species, such as the unusual antimicrobial susceptibility in *F. russii* we have found (Goldstein *et al*., 1999) or the coccoid morphology in *F. perfoetens*, which created its original name *Coccobacillus perfoetens.*

Whereas the ITS segments we have sequenced were found to consist of a 16S rRNA-gene segment of constant 112 bp-length, the lengths of the spacer itself (115-262 bp) and of the 23S rRNA-gene part (462-518 bp) have variations specific for species or subspecies (**Table 1,** columns 8 and 9). As a possible new tool for bacterial detection, primers could be designed from corresponding flanking and conserved regions and used to identify fusobacteria without further restriction or sequencing.

In conclusion, the ITS spacer region is being increasingly used as an important tool for classification and differentiation of bacterial species. Our study is the first to provide this sequence information for all species of an obligately anaerobic genus. Its higher resolution resolves some of the current problems in molecular taxonomy. Some discrepancies exist, however, and a synergism between phenotypical and genotypical approaches is still needed.

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Fig 1. Representative gel-electrophoretical ITS amplification patterns of fusobacterial species.

Figure 1:

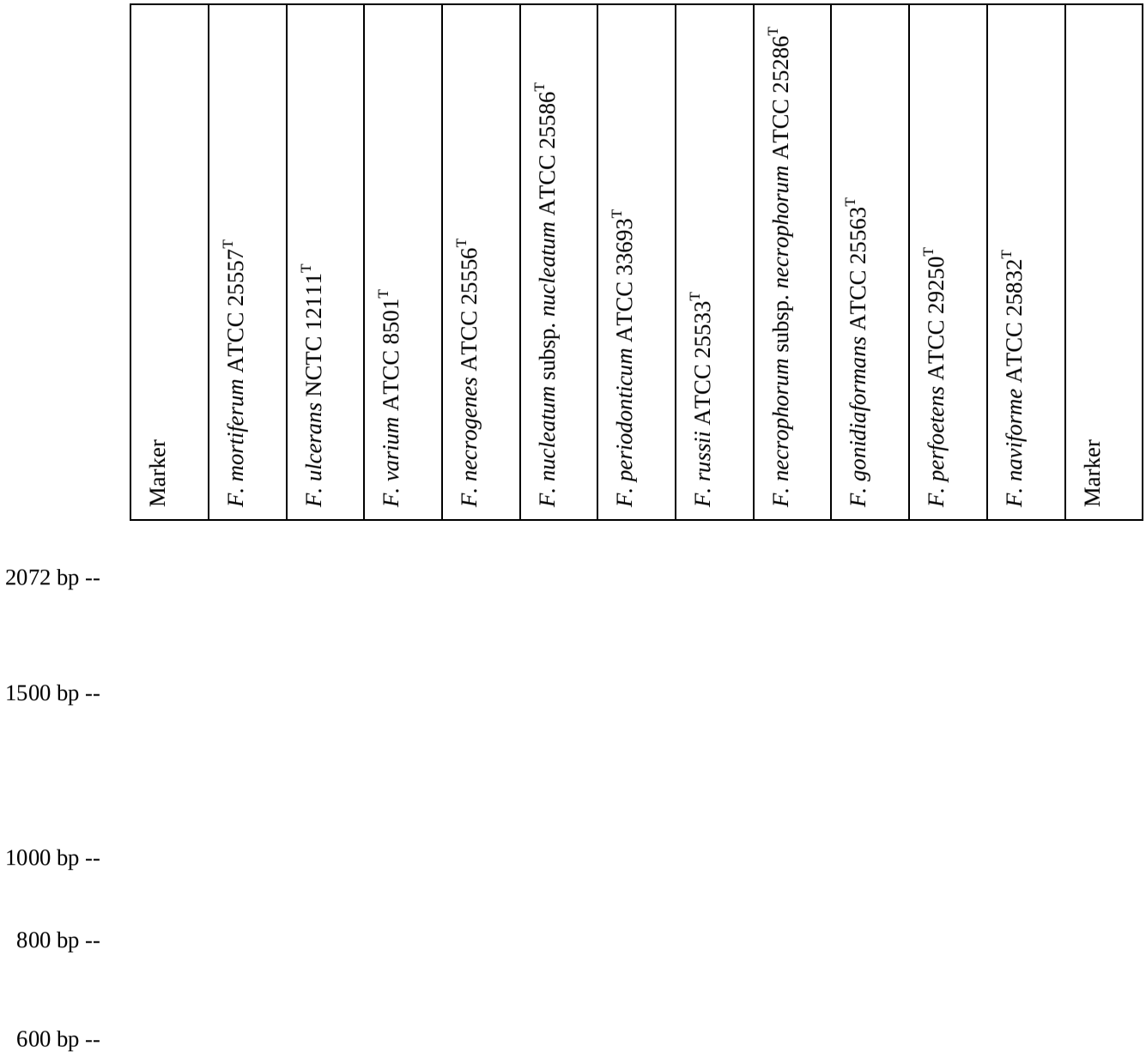
Marker
F. mortiferum ATCC 25557T
F. ulcerans NCTC 12111T
F. varium ATCC 8501T
F. necrogenes ATCC 25556T
F. nucleatum subsp. nucleatum ATCC 25586T
F. periodonticum ATCC 33693T
F. russii ATCC 25533T
F. necrophorum subsp. necrophorum ATCC 25286T
F. gonidiaformans ATCC 25563T
F. perfoetens ATCC 29250T
F. naviforme ATCC 25832T
Marker
2072 bp --
1500 bp --
1000 bp --
800 bp --
600 bp --

Fig 2. Representative ITS region deduced from the type species *F. nucleatum* subsp. *nucleatum* (ATCC 25586^{T}). Highlighted: conserved regions with *Eco*RI restriction site underlined. Bold: 16S rRNA-23S rRNA spacer.

Figure 2:

16S rRNA gene

001GAAGTAGCAGGCCTAACCGTAAGGAGGGATGTTCCGAGGGTGTGATTAGC
051GATTGGGGTGAAGTCGTAACAAGGTATCCGTACGGGAACGTGCGGATGGA
101TCACCTCCTTTC**TAAGGAGAATGTGTCTTTCTCTATTCTATTGGTAATGT**
151**TCTTACATTACTTCTGAACATTGGAAACTATATAGTAGAACAAACAAGAA**
201**AAAAATTAACTCTAAACAATTTCTTTAGAGTTAGCTTGNCAAAAAAT**AGG
251TTAAAATAATTAAGGGCACACAAAGGATGCCTAGGTAGTAAGAGCCGATG
301AAGGACGTGGTAAGCCTGCGATAAGCCTAGATAAGTTGCAATCGAACGTA
351AGAGTCTAGGATTTCCGAATGGAGCAATCTATTAAGATGGAGTCTTAATA
401CGAAAGAGGGAACCGCGTGAACTGAAACATCTAAGTACCGCGAGGAAAAG
451AAAGTAAAAACGATACCCAAAGTAGCGGCGAGCGAACTGGGTCAAGCCTA
501AACCTTAAATATGTCAAGGATACAGCCGTTGTATTTAAGGGGTAGAGGGA
551CAAAGTAGTGAAGAACTGTAAGATATTCAATATAGTGTATTGATGAATTA
601GAATTGTCTGGAAAGATGAACCGCAGAAGGTGAAAGTCCTGTATAAGTAA
651ATCCTTACACATATAACTTTGCTCCCAAGTAACATGGAACACGAGGAATT
701CTGTGTGAATCAGTGAGGACCAAATCTCATAAGGCTAAATACTCT

23S rRNA gene

Fig 3. Phylogram (neighbour-joining method) showing the genetic relationships among fusobacterial species based on the DNA sequences of their short 16S-23S rDNA spacer regions (GenBank accession numbers included). *F. prausnitzii* strains ATCC 27766 and ATCC 27768^{T} were used as an outgroup.

Figure 3:

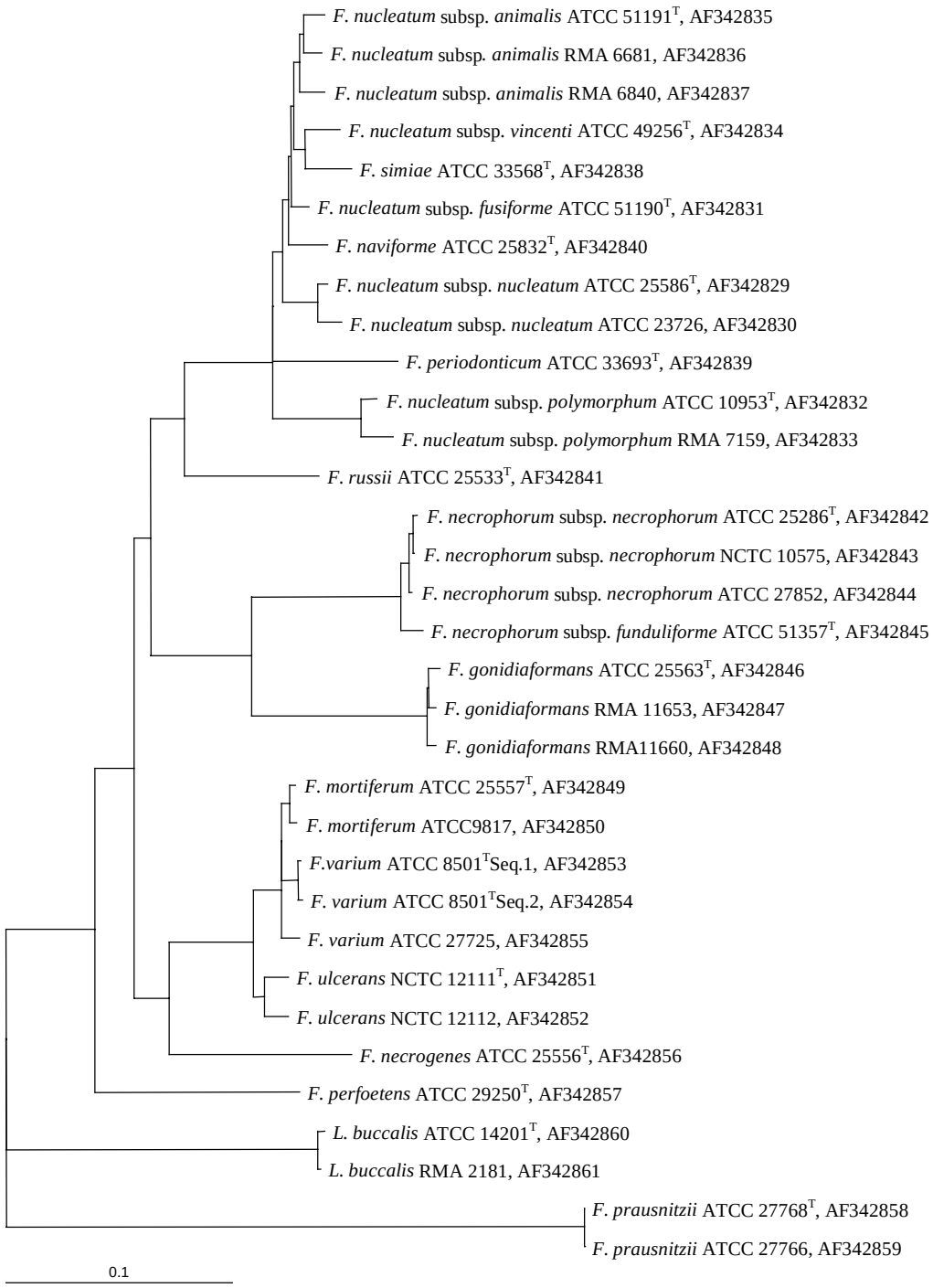
F. nucleatum subsp. animalis ATCC 51191T, AF342835
F. nucleatum subsp. animalis RMA 6681, AF342836
F. nucleatum subsp. animalis RMA 6840, AF342837
F. nucleatum subsp. vincenti ATCC 49256T, AF342834
F. simiae ATCC 33568T, AF342838
F. nucleatum subsp. fusiforme ATCC 51190T, AF342831
F. naviforme ATCC 25832T, AF342840
F. nucleatum subsp. nucleatum ATCC 25586T, AF342829
F. nucleatum subsp. nucleatum ATCC 23726, AF342830
F. periodonticum ATCC 33693T, AF342839
F. nucleatum subsp. polymorphum ATCC 10953T, AF342832
F. nucleatum subsp. polymorphum RMA 7159, AF342833
F. russii ATCC 25533T, AF342841
F. necrophorum subsp. necrophorum ATCC 25286T, AF342842
F. necrophorum subsp. necrophorum NCTC 10575, AF342843
F. necrophorum subsp. necrophorum ATCC 27852, AF342844
F. necrophorum subsp. funduliforme ATCC 51357T, AF342845
F. gonidiaformans ATCC 25563T, AF342846
F. gonidiaformans RMA 11653, AF342847
F. gonidiaformans RMA11660, AF342848
F. mortiferum ATCC 25557T, AF342849
F. mortiferum ATCC9817, AF342850
F.varium ATCC 8501TSeq.1, AF342853
F. varium ATCC 8501TSeq.2, AF342854
F. varium ATCC 27725, AF342855
F. ulcerans NCTC 12111T, AF342851
F. ulcerans NCTC 12112, AF342852
F. necrogenes ATCC 25556T, AF342856
F. perfoetens ATCC 29250T, AF342857
L. buccalis ATCC 14201T, AF342860
L. buccalis RMA 2181, AF342861
F. prausnitzii ATCC 27768T, AF342858
F. prausnitzii ATCC 27766, AF342859
0.1

Table 1. Number and length of different amplificons as well as 16S/spacer/23S composition of ITS amplificons in fusobacterial species and relatives.

Table 1:

Species	Strain	No. of bands	Short version [bp]	Long version [bp]	Dimer (facultative) [bp]	16S part [bp]	Spacer [bp]	23S part [bp]
F. mortiferum	ATCC 25557^T	3-4	830	1050	2100	112	168	501
F. mortiferum	ATCC 9817	3-4	830	1050	2100	112	168	501
F. ulcerans	NCTC 12111^T	3-4	830	1050	2100	112	171	496
F. ulcerans	NCTC 12112	3-4	830	1050	2100	112	171	496
F. varium	ATCC 8501^T	3-4	830	1050	2100	112	168	498
F. varium	ATCC 27725	3-4	830	1050	2100	112	168	498
F. necrogenes	ATCC 25556^T	3-4	880	1080	2200	112	208	490
F. nucleatum subsp. *nucleatum*	ATCC 23726	3-4	800	1050	1900	112	124	496
F. nucleatum subsp. *nucleatum*	ATCC 25586^T	3-4	810	1050	1950	112	135	498
F. nucleatum subsp. *fusiforme*	ATCC 51190^T	3	800	1050	-	112	123	496
F. nucleatum subsp. *polymorphum*	RMA 7159^T	1	850-920	-	-	112	151	479
F. nucleatum subsp. *polymorphum*	ATCC 10953^T	1	850-920	-	-	112	151	479
F. nucleatum subsp. *vincenti*	ATCC 49256^T	3	800	1050	-	112	121	496
F. nucleatum subsp. *animalis*	RMA 6840	3	810	1050	-	112	132	497
F. nucleatum subsp.*animalis*	RMA 6681	3	810	1050	-	112	132	498
F. nucleatum subsp.animalis	ATCC 51191^T	3	810	1050	-	112	132	497
F. simiae	ATCC 33568^T	3	810	1050	-	112	132	497
F. periodonticum	ATCC 33693^T	1	900	-	-	112	186	498
F. naviforme	ATCC 25832^T	1	800	-	1900	112	123	498
F. russiii	ATCC 25533^T	1-2	800	-	1900	112	115	497
F. necrophorum subsp. *funduliforme*	ATCC 51357^T	1	830	-	-	112	139	495
F. necrophorum subsp. *necrophorum*	ATCC 27852	1	830	-	-	112	139	495
F. necrophorum subsp. *necrophorum*	NCTC 10575	1	830	-	-	112	139	495
F. necrophorum subsp. *necrophorum*	ATCC 25286^T	1	830	-	-	112	139	495
F. gonidiaformans	RMA 11660	3-4	800	1000	1900	112	168	462
F. gonidiaformans	RMA 11653	3-4	800	1000	1900	112	168	462
F. gonidiaformans	ATCC 25563^T	3-4	800	1000	1900	112	168	462
F. perfoetens	ATCC 29250^T	1	830	-	-	112	115	518
L. buccalis	RMA 2181	3	810	1100	-	112	108	496
L. buccalis	ATCC 14201^T	3	810	1100	-	112	108	496
F. prausnitzii	ATCC 27766	3	950	1150	-	112	262	480
F. prausnitzii	ATCC 27768^T	3	950	1150	-	112	262	480
S.moniliformans	ATCC 14647^T	3-4	900	1100	2300	112	207	497
S. termitidis	ATCC 33386^T	3-4	810	1000	2000	112	113	447

Data for *A. actinomycetemcomitans* ATCC 33384^T, *Bacteroides forsythus* ATCC 43037^T, *Escherichia coli* ATCC 25922, *Eubacterium lentum* ATCC 43055, *Porphyromonas gingivalis* ATCC 33277^T, and *Streptococcus mutans* ATCC 25175^T, used for contrast, are not shown.