A Systematic Review of the Root Canal Microbiota Associated with Apical Periodontitis: Lessons from Next-Generation Sequencing

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Next-generation sequencing (NGS) has now been applied for a decade to characterize the microbiota composition of infected dental root canals associated with apical periodontitis. Here, the study aims at systematically and critically reviewing these reports within the outcome of interest selected; the microbiota composition in different endodontic infection types. Standard methodological guidelines as stated by the PRISMA and the Joanna Briggs Institute are followed, including a risk of bias assessment. A literature search is conducted using the PubMed Advanced-Search Builder on April 8, 2019; only original research articles that investigated the microbiota of infected root canals by means of NGS are screened. Among the 26 articles initially identified, 18 are included and evaluated for the following parameters; sampling protocol, sequencing strategy, and microbiota composition. The endodontic infections include primary apical periodontitis (PAP), secondary apical periodontitis (SAP), and apical abscess (AA). All infection types are associated with a highly diverse microbiota. Although some taxa appear differentially abundant between PAPs, SAPs, and AAs, no evident clustering of the microbiota by infection type is observed. These studies collectively formulate a comprehensive map of the taxa associated with endodontic infections and provide evidence of compositionally unspecific, yet abundance differentiates, community profiles according to clinical diagnosis.

1. Introduction

Apical periodontitis is an inflammatory condition of dental peri-radicular tissues caused by bacteria invading the dental pulp and the root canal system.\[3\] Bacterial invasion of the root canals (termed an “endodontic” infection) occurs as a sequel to caries, trauma, or periodontal diseases and invariably leads to the necrosis of the infected tissues.\[2\] Complex bacterial communities rapidly colonize necrotized root canals, reach the connective tissues surrounding the teeth through the apical foramen, and induce a periapical lesion termed “primary apical periodontitis” (PAP).\[3,4\] PAP lesions can be cured by an endodontic treatment that primarily aims at eradicating the infecting bacteria from root canals. However, unsuccessfully treated root canals harbor persistent bacterial communities that perpetuate a periapical lesion referred to as “secondary apical periodontitis” (SAP).\[5–7\] According to clinical reports, such cases are remarkably high among Western populations. Around 41–59% of individuals bear at least one endodontic treatment, and 24–65% of these endodontically treated teeth remain associated with SAP lesions.\[8–12\] PAP and SAP lesions may unfavorably evolve toward the formation a purulent collection termed an “apical abscess” (AA). All these endodontic infection types (PAPs, SAPs, and AAs) cause inflammation, alveolar bone destruction, and may exacerbate into potentially life-threatening infections.\[13–15\] Furthermore, there is growing evidence that demonstrates associations between chronic apical periodontitis lesions and systemic diseases such as diabetes mellitus or cardiovascular diseases.\[16,17\] In this context, considerable efforts are invested in the characterization of the root microbiota associated with PAP and SAP lesions. Such knowledge is expected to provide a framework to improve current therapeutic regimens and reduce the incidence of persistent endodontic infections.

In recent years, high-throughput DNA sequencing technologies, so-called next generation sequencing (NGS), have become the method of choice to comprehensively characterize microbiotas from human sites, including the oral cavity.\[18,19\] Bacterial identification most commonly relies on the PCR amplification and subsequent sequencing of the 16S ribosomal RNA...
(16S rRNA) gene. Ubiquitous in bacteria, this gene advantageously combines highly conserved regions, along with nine variable regions that differ considerably among bacterial taxa and that are valuable targets for bacterial identification. A typical analysis pipeline starts by clustering these variable regions based on sequence similarity into a single representative sequence of a so-called operational taxonomic unit (OTU). OTUs are finally assigned to a taxonomic identity by comparison with 16S rRNA gene databases. Currently (July 2019), 20 160 bacterial 16S rRNA sequences are publicly available on the “NCBI 16S ribosomal RNA project” and the “Human Oral Microbiome Database” (HOMD) comprises 693 sequences of 16S rRNA genes representative of oral taxa only. NGS technologies have now been employed for almost 10 years to characterize the bacterial communities of infected root canals associated with apical periodontitis. Throughout these studies different sampling techniques have been employed, different sequencing technologies utilized and different types of root canal infections investigated. This variety of parameters may render difficult to grasp a synthetic overview of the field and to outline the main trends in the composition of the root microbiota associated with apical periodontitis.

Here, we aimed at systematically and critically reviewing reports that have investigated the microbiota of root canals associated with apical periodontitis by means of NGS technologies. The outcome of interest evaluated was the microbiota composition in PAPs, SAPs, and AAs. Specifically, the parameters evaluated in the included articles were the type of endodontic infections, the bacterialsampling methodology, the sequencing technology, and the microbiota composition. It is anticipated that this systematic review would accelerate the display of previously published results and potentially highlight areas where additional research would be warranted.

2. Experimental Section

The methodology followed for completion of this systematic review was in accordance with the PRISMA statement and Cochrane guidelines for systematic reviews (version 5.1.0 http://handbook-5-1.cochrane.org/).

2.1. Literature Search

A literature search was performed using the PubMed Advanced Search Builder on April 8, 2019 with the aim of identifying original research articles that investigated the microbiota of infected root canals by means of NGS. The search strategy included three distinct blocks of keywords. Block 1: “illumina” OR “pyrosequencing” OR “next-generation sequencing” OR “ngs” OR “high-throughput sequencing.” Block 2: “bacterial composition” OR “microbiome” OR “microbiota” OR “metagenomics” OR “microbial communities” OR “16S ribosomal RNA” OR “16s rRNA.” Block 3: “endodontic infection” OR “root canal” OR “dental pulp cavity” (MeSH term) OR “periapical periodontitis” (MeSH term) OR “apical periodontitis” OR “primary apical periodontitis” OR “secondary apical periodontitis.” The final query combined the three keyword blocks each separated by AND to collect publications that incorporate the three blocks of interest.
2.2. Filtering Criteria

Article abstracts obtained from the literature search were screened independently by D.M., G.B., and K.A. using the following criteria. Only original articles in English were considered. Reviews, articles non-related to endodontic infections, or not employing NGS technologies for bacterial identification were excluded from further analysis. The final decision to include/exclude articles was consensually made among the four researchers.

2.3. Extraction of Data of Interest and Quality Assessment

The data of interest that were extracted from the original publications were 1) the type of endodontic infection, 2) the bacterial sampling methodology, 3) the sequencing technology, and 4) the microbiota composition. In accordance with the Joanna Briggs Institute Reviewer’s Manual – 2017 (https://joannabriggs.org/), the articles included were critically appraised independently by three authors; D.M., G.B., and K.A. (Table S1, Supporting Information). In addition, Table 3 sums up the most relevant suspected biases and provides a support for judgment each time a potential risk of bias was identified. Potential biases were considered as factors that affect the outcome of interest of this review, i.e., the characterization of the root microbiota associated with PAPs, SAPs, or AAs. Studies were graded into three categories; low risk (unlikely to affect the outcome of interest), some concerns (unpredictable effect on the outcome of interest), and high risk (likely to affect the outcome of interest).[24]

3. Results

A total of 26 articles were identified in the initial literature search. Among the 21 that passed the initial screening, three were not considered eligible for this review. Among these three, one was discarded because it was analyzing the microbiota of deciduous teeth, and two others because bacterial sampling was performed from extra-radicular tissues (granulomas/cysts).[25–27] Eventually, 18 met the specified criteria and were original articles that studied the microbiota composition of infected root canals associated with apical periodontitis by means of NGS (Figure 1). Table 1 presents the main characteristics of the studies selected.
Table 1. Overview of the key characteristics of the included articles.

| Study | Location | NGS technology | 16S rRNA variable regions | Similarity cutoff for OTU clustering | Total OTUs clustered | Database for taxonomic assignment | Types of endo. infections | Number of samples | Presence/size of apical lesion | Sampling methodology |
|-------|----------|----------------|--------------------------|--------------------------------------|----------------------|----------------------------------|--------------------------|---------------------|--------------------------|---------------------|
| Li et al. (2012) | University of Maryland, College Park, MD | 454 GS-FLX pyrosequencing | 16S rRNA - V1-V2 | Unspecified similarity cutoff | 454 GS-FLX pyrosequencing | HOMD and RDP | PAP and SAP without group assignment distinction | PAP (6)/SAP (1) | AP present 3–10 mm | In vivo - paper points |
| Santos et al. (2013) | Estació de Sa University, Rio de Janeiro, Brazil | 454 GS-FLX pyrosequencing | 16S rRNA - V1-V2 | 97% similarity | 325 | 454 GS-FLX pyrosequencing | PAP and AA | 10 | AP present/no size mentioned | In vivo: paper points and abscess aspiration |
| Siqueira et al. (2016) | Estació de Sa University, Rio de Janeiro, Brazil | 454 GS-FLX pyrosequencing | 16S rRNA - V1-V3 | 97% similarity | 606 | RDP and unidentified OTU in HOMD | PAP and SAP | 23 | AP present/no size mentioned | In vivo: paper points and abscess aspiration |
| Hsiao et al. (2017) | University of Maryland, College Park, MD | 454 GS-FLX pyrosequencing | 16S rRNA - V1-V2 | 97% similarity | 803 | SILVA and RDP | PAP and SAP | PAP (10)/SAP (8) | AP present/no size mentioned | Ex vivo: cryo-pulverized roots |
| Özok et al. (2018) | University of Amsterdam, Academic Center for Dentistry Amsterdam, Netherlands | 454 GS-FLX pyrosequencing | 16S rRNA - V1-V3 | 97% similarity | 803 | SILVA and HOMD | PAP and SAP | 803 | AP present/no size mentioned | In vivo: paper points |
| Hong et al. (2019) | Seoul National University, Seoul, South Korea | 454 GS-FLX pyrosequencing | 16S rRNA - V1-V3 | 97% similarity | 803 | SILVA and HOMD | PAP and SAP | 803 | AP present/no size mentioned | Ex vivo: cryo-pulverized roots |

(Continued)
| Study | Anderson et al. (2013) | Vengerfeldt et al. (2014) | Tzanetakis et al. (2015) | Gomes et al. (2015) | Siqueira et al. (2016) | Persoon et al. (2017) |
|-------|------------------------|--------------------------|-------------------------|---------------------|-----------------------|----------------------|
| Types of endo. infections | SAP | PAP, SAP, and AA | PAP and SAP | PAP associated with marginal periodontal lesion. | SAP | PAP |
| Number of samples | 40 | PAP (5), SAP, (3) and AA (4) | PAP (24) and SAP (24) | 15 | 10 | 23 |
| Presence/size of apical lesion | AP present/no size mentioned | AP present/no size mentioned | AP present/no size mentioned | Presence of AP either ≤ or >2 mm on Rx | AP present on CBCT, no size mentioned | AP present/no size mentioned |
| Sampling methodology | In vivo: paper points | In vivo: paper points | In vivo: paper points | In vivo: paper points | Ex vivo: roots associated with granuloma (7) and cysts (3) extracted through apical surgery | Ex vivo: cryo-pulverized roots (distinguished between coronal and apical segments) |

| Study | Keskin et al. (2017) | Sanchez-Sanhueza et al. (2018) | Iriboz et al. (2018) | Zandi et al. (2018) | Bouillaguet et al. (2018) | Qian et al. (2019) |
|-------|---------------------|-------------------------------|-------------------|----------------------|--------------------------|-------------------|
| Location | Ondokuz Mayis University, Samsun, Turkey | School of Dentistry, University of Concepcion, Concepcion, Chile | Marmara University, Istanbul, Turkey | University of Oslo, Institute of Clinical Density, Oslo, Norway | Geneva University Hospital, Geneva, Switzerland | Shanghai, China |
| NGS technology | 454 GS-FLX pyrosequencing | Illumina MiSeq | Illumina MiSeq | 454 GS-FLX pyrosequencing | Illumina MiSeq | Illumina MiSeq |
| 16S rRNA variable regions | 16S rRNA: V4 | 16S rRNA: V3–V4 | 16S rRNA: V3–V4 | 16S rRNA: V3–V5 | 16S rRNA: V3–V4 | 16S rRNA: V3–V4 |
| Similarity cutoff for OTU clustering | 97% similarity | Unspecified similarity | 97% similarity | 97% similarity | 97% similarity | 97% similarity |
| Total OTUs clustered | Not specified | 86 | 85 | 152 | 347 | Unclear report (ranging from 918 to 4436?) |
| Database for taxonomic assignment | NCBI | GG | SILVA | SILVA and HOMD | EzBioCloud and HOMD | GG |
| Types of endo. infections | PAP and SAP | SAP | PAP | SAP | PAP and SAP | PAP and SAP |
| Number of samples | PAP (20) and SAP (20) | 24 | 20 | 10 | PAP (21) and SAP (22) | PAP (23) and SAP (8) |
| Presence/size of apical lesion | Presence of AP, PAI score ≥3 included | Presence of AP, PAI score ≥3 included | AP present/no size mentioned | AP present/no size mentioned | AP present/no size mentioned | Presence of AP between 2 and 8 mm on Rx |
| Sampling methodology | Ex vivo: cryo-pulverized roots | In vivo: paper points | In vivo: paper points | In vivo: paper points | Ex vivo: intraradicular content collected with endodontic files | Ex vivo: cryo-pulverized roots |

AP; apical periodontitis.
3.1. Types of Endodontic Infections Investigated

In all the studies included, the diagnosis of endodontic infections studied could be categorized as either “primary apical periodontitis” (PAP), “secondary apical periodontitis” (SAP), or “apical abscess” (AA). Consistently across these studies, PAPs were defined by the presence of a periapical lesion on radiographs associated with an untreated root canal, SAPs by a lesion present despite a root canal treatment, and AAs by a collection of pus observed clinically. Whereas some articles focused their investigation on one of these infection types, other reports included several infection types and compared the microbiota. One study evaluated the root microbiota of PAP lesions associated with concomitant marginal bone loss, a specific pathology termed “endoperio” lesion. Overall, PAP lesions have been evaluated in 13 articles, SAP lesions in ten articles and AAs in three articles (Table 1). Eight studies (8/18) additionally documented the symptomatology, without, however, always including this parameter in their further analyses.

3.2. Bacterial Sampling Methodology

Sampling methodologies employed in these studies can be separated into in vivo or ex vivo. The majority of the studies (11/18) used an in vivo sampling strategy, by which the root microbiota was collected using paper points inserted into the root canal during the course of an endodontic treatment. In these cases, the teeth investigated were to be retained in the oral cavity. Seven studies (7/18) employed an ex vivo sampling methodology, in which the roots were extracted prior to sampling the microbiota. Six of these studies relied on cryo-pulverization of root sections, a technique used in sectioning the apical extremity of the root, that is then ground at liquid nitrogen temperatures in dedicated freezer mills. Figure 2 illustrates these two main bacterial sampling approaches. Bouillaguet et al. used an alternative ex vivo approach, in which the tooth was extracted, the apical segment of the root sectioned and the intra-radicular bacterial content collected using dedicated endodontic instruments. Concerning sampling in cases of AAs (3/18 studies), two of the studies employed needle aspiration of pus. Whereas Hsiao et al. used aspiration in addition to paper point sampling, Santos et al. relied exclusively on pus aspiration. However, because this review focuses on the root microbiota, AAs-related results originating from extra-radicular sampling are not reported herein.

3.3. Sequencing Strategy

The sequencing technologies employed in the studies included were 454 GS-FLX pyrosequencing (Roche) (10/18 studies) and Illumina MiSeq (7/18 studies) and HiSeq (1/18 studies; Illumina Inc.; Table 1). In all studies, bacterial taxonomic identification relied on PCR amplification of “taxa-specific” variable regions of the 16S rRNA gene. Regions V1-V2 and V3-V4 were the most frequently covered, as shown in Figure 3. Sequencing reads have been clustered into OTUs based on a 97% similarity cutoff in 13 studies, whereas four studies omitted to specify the similarity cutoff employed (Table 1). One study alternatively relied on species- and genera-specific “probes” from the Forsyth Institute
assign taxonomy. The 16S rRNA databases searched for the taxonomic assignment of the OTUs included, HOMD (9/18), SILVA (6/18), Ribosomal Database Project (RDP, 6/18), Green genes (GG, 4/18), NCBI (1/18), and EzBioCloud 16S (1/18) (Table 1).

3.4. Microbiota Composition in Infected Roots

3.4.1. Overview

Regardless of the infection type, the most abundant phyla represented (>5%) were Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria. Phyla detected at abundances <5% included Synergistetes, Spirochaetes, or TM7, and other phyla such as Tenericutes, Chloroflexi, OD1, SR1, Cyanobacteria, or Acidobacteria were detected at abundances <0.5%. Deeper analysis and assessment of lower taxonomic ranks displayed important disparities between studies. Table 2 presents an overview of the notable bacterial communities identified by the articles included. The following paragraphs summarize notable bacterial profiles organizing the main findings by type of endodontic infection.

3.4.2. Primary Apical Periodontitis

Siqueira et al. was among the first to employ NGS to investigate the microbiota composition in primarily infected root canals. They identified 187 OTUs assigned to 84 genera among which the most abundant were Fusobacterium (15%), Pseudorambacter (8%), Novosphingobium (8%), Ralstonia (6%), and Bacteroides (5%). A subsequent study by Özok et al. identified significantly more OTUs (606), including high abundances of the genera Lactobacillus, Prevotella, Fusobacterium, Actinomyces, Parvimonas, Pseudorambacter, and Porphyromonas. These results are in agreement with Persoon et al., who reported the same genera among the most abundant of the 338 OTUs detected in PAP-associated roots. Investigating PAP lesions concomitant with marginal bone loss (endo-perio lesions), Gomes et al. identified 73 phylotypes within root canals, among which the most abundant species were Enterococcus faecalis followed by Parvimonas micra, Mogibacterium timidum, Peptostreptococcus stomatis, Filactor alocis, and Fretibacterium fastidiosum. High abundances of Mogibacterium timidum and Fretibacterium fastidiosum were also reported in PAPs by Bouillaguet et al. among the 347 OTUs detected, along with Fusobacterium nucleatum, Parvimonas micra, Porphyromonas endodontalis, Prevotella oris, Dílister pneumosintes, and Streptococcus constellatus that were hallmark of PAP lesions (Table 2).

3.4.3. Secondary Apical Periodontitis

Among the first studies to have addressed the root microbiota of SAP lesions, Anderson et al. detected 741 OTUs, which were assigned to 277 genera with Streptococcus (10.9%), Prevotella (8.21%), Lactobacillus (8.06%), Kocuria (5.17%), Neisseria (3.38%), and Enterococcus (2.59%) being among the most abundant (Table 2). Subsequent investigations of Siqueira et al. identified 538 OTUs with highly abundant Fusobacterium and Pseudomonas (15%), followed by Enterococcus (2%) and then Klebsiella, Stenotrophomonas, Pseudorambacter, and Pyramibacter with important interindividual variability. Also on SAPs,
| Study                          | Li et al. (2010)                                                                 | Santos et al. (2011)                                                                                   | Siqueira et al. (2011)                                                                                   | Hsiao et al. (2012)                                                                 | Ozok et al. (2012)                                                                 | Hong et al. (2013)                                                                 |
|-------------------------------|--------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| Outcome investigated          | Observational study of the microbiota from PAPs and SAPs indiscriminately.      | Comparison of the microbiota from PAP-associated roots (asymptomatic) and AAs (symptomatic)        | Observational study of the microbiota from apical segments associated with PAP                       | Comparison of the microbiota from oral swabs, AA-associated root canals and pus aspiration from AA | Comparison of the root microbiota from coronal and apical root segments associated with PAPs | Comparison of the root microbiota from PAPs and SAPs |
| Main findings                 | 179 Genera belonging to 13 phyla were identified. Many low-abundant taxa detected with undefined clinical implications. Six low-abundant phyla reported for the first time: Tenericutes (0.6%), Deinococcus-Thermus (0.16%), Chloroflexi (0.10%), Cyanobacteria (0.01%), OD1 (0.003%), and Acidobacteria (0.001%). | 67 Genera belonging to 13 phyla detected. 20 genera were exclusively detected in pus from AAs and 18 in PAPs. Overall 18% of the 165 OTUs detected in both pus and PAP-associated root canal. | 84 Genera belonging to ten phyla detected. The majority of the OTUs detected were low-abundant. Important interindividual variation in the composition of the apical microbiota was disclosed. | 11 Phyla detected. Lower bacterial diversity in AAs root canals and pus than in oral swabs. Streptococcus was the most abundant genus in oral swabs, Prevotella, and Fusobacterium were most abundant in AAs root canals and pus. | 24 Bacterial phyla detected, Proteobacteria more abundant in the apical, and Actinobacteria more abundant in coronal segments. Apical segments displayed higher diversity than the coronal segments. | 148 Genera belonging to, and 10 phyla detected. No significant differences in bacterial composition between the two infection types. |
| Main phyla (max. top 6)       | Bacteroidetes (59.5%), Firmicutes (19.9%), Actinobacteria (4.8%), Fusobacteria (3.5%), Proteobacteria (3.2%), Spirochetes (2.3%). | In PAP samples: Firmicutes (59%), Bacteroidetes (14%), Actinobacteria (10%), Fusobacteria, Proteobacteria, Spirochetes. | Proteobacteria (43%), Firmicutes (25%), Fusobacteria (16%), Actinobacteria (9%), Bacteroidetes (5%), Prevotella, Synergistetes. | Firmicutes (48%), Bacteroidetes, Fusobacteria, Proteobacteria, Actinobacteria, Synergistetes. | Firmicutes (48%), Actinobacteria (30%), Bacteroidetes (12%), Acidobacteria, BRC1, Chlamydiae. | In both infection types: Bacteroidetes (29.6%), Firmicutes (23.2%), Actinobacteria (10.5%), Fusobacteria (13.1%), Proteobacteria (8.8%), Synergistetes (6.3%). |
| Main genera/species (max. top 12) | Pseudoalteromonas, Fusobacterium, Streptococcus, Ralstonia, Marinimonas, Acinetobacter, Pseudomonas, Prevotella, Olsenella, Micrococcus, Paracoccus, Aeromonas. | Most abundant genera in PAPs: Phocaeicola (12.5%), Eubacterium (12%) and Pseudoramibacter (10%). The most abundant genera from pus sampling: Fusobacterium (19%), Porphyromonas (11%) and Peptostreptococcus (10%). | Fusobacterium (15%), Pseudoramibacter (8%), Novosphingobium (8%), Ralstonia (6%), Bacteroides (5%). | Fusobacterium, Prevotella, Eubacterium, Granulicatella, Erysipelotrichaceae, Streptococcus, Porphyromonas, Afpia, Phocaeicola, Veillonella, Porphyromonas, Gemella, Pyramidobacter. | Lactobacillus (14.3%), Actinomyces (11.9%), Streptococcus (0.4%), unclassified Actinobacteria (6.9), Prevotella (6.1%), Parvimonas (3.4%), Oder Bacterioidales (1.1%), Veillonellaceae (2.5%), Fusobacterium (2%), Peptostreptococcus (2%), Porphyromonas and Prevotella. | In PAPs: Prevotella, Propionibacterium, and Pyramibacter. |
|                              |                                                                                 |                                                                                                    |                                                                                                    |                                                                                                    |                                                                                                    |                              |

(Continued)
Table 2. Continued.

| Study                        | Outcome investigated                                                                 | Main findings                                                                 | Main phyla (max. top 6)                                                                 | Main genera/species (max. top 12)                                                                 | No assessment at the phylum level                                                                 |
|------------------------------|--------------------------------------------------------------------------------------|-------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|
| Anderson et al. (2013)       | Comparison of the root microbiota from symptomatic and asymptomatic SAPs             | Symptomatic SAPs displayed more Firmicutes and Fusobacteria than asymptomatic SAPs. In turn, asymptomatic SAPs exhibited more Proteobacteria and Actinobacteria. | Firmicutes (29.9%), Proteobacteria (26.1%), Actinobacteria (22.72%), Bacteriodetes (13.31%) and Fusobacteria (4.55%). | Streptococcus (10.9%), Prevotella (8.21%), Lactobacillus (8.06%), Kocuria (5.17%), Neisseria (3.38%), Enterococcus (2.59%), Acinetobacter, Atopobium, Rothia, Pseudomonas, Propionibacterium, Schlegelella. | No relative abundances were reported for any of the genera detected. |
| Vengerfeldt et al. (2014)     | Comparison of the root microbiota from PAPs, SAPs and AAs                             | All infection types displayed highly diverse communities. Communities composition displayed interindividual variability. E. faecalis was found only in SAPs. One AA sample displayed a significantly high proportion (47%) of Proteobacteria, mainly composed of Janthinobacterium lividum. | In all infection types: Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria, Proteobacteria, Synergistetes. | PAPs enriched with: Bacteroidaceae_unclassified, Peptostreptococcaceae [G-1] sp oral taxon 272, Peptostreptococcus stomatis, Filifactor alocis, and Fretibacterium fastidiosum. | In PAP-associated roots: Enterococcus faecalis, Parvimonas micros, Bacteroidaceae [G-1] sp oral taxon 113, Megabacterium timidum, Peptostreptococcus stomatis, Filifactor alocis, and Fretibacterium fastidiosum. |
| Tzanetakis et al. (2015)      | Comparison of the root microbiota from PAPs and SAPs. In each case discriminating between symptomatic and asymptomatic SAPs. Bacteroidetes was the most abundant phylum in both infection types. Symptomatic and asymptomatic infections displayed different bacterial compositions. SAPs were significantly enriched with Proteobacteria and Tenericutes as compared to PAPs. 14 genera were differentially abundant between PAPs and SAPs. | Overall, the genera Firmicutes, Actinobacteria, and Fusobacteria were the most abundant. Associations were found between periapical lesions ≤ 2 mm and Fusobactribus sp oral taxon 041. | In all infection types: Firmicutes (36.2%), Actinobacteria (8.1%), Synergistetes (7.4%), Fusobacteria (7.4%), Proteobacteria (9.2%). | In PAP-associated roots: Firmicutes (75.09%), Proteobacteria (7.85%), Actinobacteria (7.01%), Proteobacteria (6.77%). | In PAP-associated roots: Firmicutes (81%), Proteobacteria (15%) and Actinobacteria (8%). |
| Gomes et al. (2015)           | Comparison of the root microbiota from PAPs and periodontal pockets in cases of endo-perio lesions, prior to and after endodontic disinfection | Firmicutes was the most abundant Phylum in all sampling sites. Associations were found between periapical lesions ≤ 2 mm and Desulfovibulus sp oral taxon 041. | Proteobacteria (46%), Fusobacterium (18%), Actinobacteria (15%) and Actinobacteria (8%). | Fusobacterium and Pseudomonas (15 %), Klebsiella, Stenotrophomonas, Pseudoramibacter, Pyridostibacter, Enterococcus. | No correlations between bacteriaes and mycobiones identified no correlations. |
| Siqueira et al. (2016)        | Observational study of the root microbiota from apical segments as associated with SAPs. | 103 genera belonging to 11 phyla were detected. Overall, the genera Fusobacterium and Pseudomonas were the most dominant. Enterococcus was found in 4 cases, always in relatively low abundance. | Proteobacteria (46%), Firmicutes (18%), Fusobacterium (15%) and Actinobacteria (8%) | Fusibacterium and Pseudomonas (15 %), Klebsiella, Stenotrophomonas, Pseudoramibacter, Pyridostibacter, Enterococcus. | No assessment at the phylum level. |
| Persoon et al. (2017)         | Comparison between the root microbiota and mycobioni from PAPs, further distinction between coronal and apical root segments. Coronal and apical root segments exhibited similar microbiome and mycobioni profiles. When fungi present canals enriched with Actinomyces, Bifidobacterium, Lactobacillus, Propionibacterium and Streptococcus. No correlations between bactereiomes and mycobiones identified no correlations. | Coronal and apical root segments exhibited similar microbiome and mycobioni profiles. When fungi present canals enriched with Actinomyces, Bifidobacterium, Lactobacillus, Propionibacterium and Streptococcus. No correlations between bactereiomes and mycobiones identified no correlations. | No assessment at the phylum level. | No assessment at the phylum level. | No assessment at the phylum level. |

(Continued)
Table 2. Continued.

| Study          | Keskin et al. (2017) | Sanchez-Sanhueza et al. (2018) | Iriboz et al. (2018) | Zandi et al. (2018) | Bouillaguet et al. (2018) | Qian et al. (2019) |
|----------------|----------------------|--------------------------------|----------------------|----------------------|--------------------------|-------------------|
| **Outcome investigated** | Comparison of the root microbiota from PAPs and SAPs | Observational study of the microbiota from SAPs | Comparison of the microbiota from PAPs prior to and after endodontic disinfection | Comparison of the microbiota from SAPs prior to and after endodontic disinfection | Comparison of the microbiota from PAPs and SAPs | Comparison of the microbiota from PAPs, SAPs, oral swabs and healthy vital teeth |
| **Main findings** | 160 genera belonging to 15 phyla were detected. PAPs and SAPs displayed no significant differences in microbiota composition. | Most OTUs belonged to the phylum Proteobacteria. Higher microbiota diversity was observed in larger periapical lesions was reduced in symptomatic patients. | Streptophyta species were abundantly detected before and after treatment. The ratio Streptophyta correlated negatively with the chances of successful bacterial elimination. | 125 bacterial species belonging to 8 genera and 9 phyla were detected. | 177 genera belonging to 18 phyla detected. Microbiotas were differentially abundant in each infection type. Co-occurrence analysis demonstrated microbial interactions specific to each infection type. | Highly abundant microbiota identified from all samples, 25 phyla detected in total. Authors report higher bacterial richness and diversity in healthy vital pulps than in PAP and SAP samples. |
| **Main phyla (max. top 6)** | Proteobacteria (33.4%), Firmicutes (32.3%), Bacteroidetes (26.3%), Fusobacteria (4.2%), and Actinobacteria (2.9%). | In order of decreasing abundances: Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Tenericutes and Fusobacteria. | Bacteroidetes (29.2%), Tenericutes (34.3%) | Prior to disinfection: Firmicutes (47%), Bacteriodes (14%), Proteobacteria (12%), Actinobacteria (9%), Synergistetes (4%). | Firmicute (36% PAP/48.4% SAP), Bacteriodes (23.8% PAP/9.5% SAP), Actinobacteria (6.4% PAP/23.4% SAP), Fusobacteria (16% PAP/5.6% SAP), Synergistetes (9.9% PAP/4.5% SAP), Proteobacteria (2.4% PAP/4.8% SAP). | Firmicute (25.6% PAP/26.2% SAP), Bacteriodes (26.6.8% PAP/17.3% SAP), Actinobacteria (11% PAP/13.6% SAP), Fusobacteria (4.2% PAP/8.6% SAP), Proteobacteria (25.3% PAP/28.7% SAP). |
| **Main genera/species (max. top 12)** | Prevotella (PAP 23.5% / SAP 15.7%), Porphyromonas (16.5% mean PAP-SAP), Neisseria (13.2% mean PAP-SAP), Lactobacillus (11.7% mean PAP-SAP), Parvimonas (11.1% mean PAP-SAP), Streptococcus (PAP 9.4% / SAP 12%), Enterococcus (PAP 2% / SAP 3%), Clostridium (PAP 2% / SAP 0.1%) and Granulicatella (1% mean PAP-SAP) | No data on genera | Among the genera that varied during the endodontic disinfection (abundances before disinfection): Dialister (39.3%), Porphyromonas endodontalis (29.8%), Prevotella (21.6%), Porphyromonas endodontalis (16.4%), Tannerella (13.8%), Prevotella (6.9%), Agrobacterium (5.9%), Methylobacterium (1.5%), Corynebacterium (0.26%) | Prior to disinfection: Enterococcus (13.9%), Fusobacterium (12.7%), Streptococcus (9.8%), Actinomyces (8.2%), Desulfoviridans (5.2%), Treponema (3.6%), Prevotella (2%), Alloprevotella (0.01%) | Fusobacterium nucleatum (PAP 16%/SAP 5.3%), Enterococcus faecalis (PAP 0.01%/SAP 18.9%), Parvimonas micro (PAP 8%/SAP 2.6%), Porphyromonas endodontalis (PAP 5.7%, SAP 2%), Streptococcus constellatus (PAP 0.6%/SAP 3.5%), Slackia exigua (PAP 0.7%/SAP 1.3%), Schwartzia AF287291 (PAP 3%/SAP 3.5%), Dialister pneumosintes (PAP 3.4%/SAP 1.2%), Prevotella oris (PAP 5.7%/SAP 1.5%). | Abundances of genera/species per infection type are not clearly reported |


Zandi et al. reported a series of abundant genera among the 152 OTUs identified, such as Fusobacterium (12.7%), Streptococcus (9.8%), Actinomyces (8.2%), Desulfovibrio (5.2%), Freretibacterium (3.6%), Treponema (2.3%), or Prevotella (2%) along with Enterococcus. Although Enterococcus was present in only two of ten samples in this study, it represented the most abundant genera (13.9%). These results concur with Bouillaguet al. who also detected Enterococcus faecalis in a limited number of SAP samples (7/22), yet in abundances ranging from 17% to 99.9% (18.9% mean of all SAP samples).

3.4.4. Comparisons between PAP and SAP

PAP and SAP samples were compared in five studies. Among these, three studies reported differentially abundant microbiotas between PAPs and SAPs. For instance, Tzanetakis et al. observed that SAPs were enriched with the phyla Proteobacteria and Tenericutes, and with 14 genera including Lactobacillus, Streptococcus, and Sphingomonas. The two infection types, however, demonstrated similar diversity indices in this study (richness and evenness). Community differences between PAPs and SAPs were also observed by Bouillaguet et al., who found higher abundances of the phyla Bacteroidetes, Fusobacteria, and Spirochaetes in PAPs as compared to SAPs, that in turn were enriched with Actinobacteria. At the species level these authors observed significantly higher abundances of Fusobacterium nucleatum, Prevotella oris, Porphyromonas endodontalis, Parvimonas micra in PAPs, whereas Enterococcus faecalis was found significantly more abundant in SAPs. Notably, this study reported a decrease in bacterial diversity in SAPs as compared to PAPs, which was likely attributable to the high proportions of E. faecalis in SAPs. In contrast, Hong et al. and Keskin et al. found no significant differences between PAP and SAP pathologies neither in terms of community composition, nor in terms of diversity.

3.4.5. Apical Abscess

In apical abscess (AA)-associated roots, Hsiao et al. detected 325 OTUs that were assigned to the genera Prevotella, Fusobacterium, and Phocaeicola at abundances >10%, Porphyromonas and Parvimonas >5%, and Streptococcus and Peptostreptococcus <5%. Vengerfeldt et al. detected only 45 OTUs in AA samples and failed to report relative abundances at the genera level. Nonetheless, these authors noticed one AA sample particularly enriched with the phylum Proteobacteria (47%), mainly attributable to Janthinobacterium lividum, which accounted for 40% of the root canal bacterial composition (Table 2).

4. Discussion

The current report provides an overview of the studies that employed NGS technologies to characterize the microbiota of infected root canals associated with PAPs, SAPs, and AAs. Focus was laid on the microbiota from root canals, as opposed to bacteria from extra-radicular tissues, because it is the one causative of apical periodontitis. It is also on the root canal microbiota that therapeutic intervention can be performed, and thus that requires our utmost understanding. Overall, compiled findings from the studies included demonstrate that all types of apical periodontitis correlated with a highly diverse microbiota. Bacterial communities appeared differentially abundant between PAPs, SAPs, and AAs, although no specific community distribution in each infection type could be observed. This conclusion enhances our understanding of the comparative microbial profiles among different endodontic infections, which is one of the open questions in the field.

Important variations were observed in the microbiota composition from different studies even when addressing comparable types of infection. Some sources of variations may be inherent to geographical or ecological factors. For instance, the microbiota data reported herein originate from 12 different countries (Table 1), and geographical factors such as water or food contaminants have been shown to influence the oral microbiota. The ecology of a root canal environment is constantly changing during the progression of an infection as oxygen is gradually depleted and tissues necrotize. Therefore, root canals sampled at different stages of an infection or divergences in clinical classifications result in inevitable variations. Sampling a long time necrotized root canal also raises the common question of whether the bacteria identified were still alive, as classical NGS protocols also detect free DNA originating from dead cells. This may indeed represent a bias in studies that invest in the effect of antimicrobials, as detection of DNA from dead cells may lead to an underestimation of the effect. In these instances, researchers may rely on techniques designed to irreversibly crosslink or degrade free DNA prior to nucleic acid extraction, and thereby ensure that the DNA analyzed downstream primarily originates from intact cells. An example is provided herein by Iriboz et al. who treated their samples with DNase I, in order to degrade free DNA prior to extraction, and further evaluated the microbiota that resisted root canal disinfection. Aside from these specific research instances, evidence suggests that the half-life of free DNA in the microbial environment of an infected root canal is very short and therefore unlikely to represent a major ratio of the DNA isolated. This half-life is attributed to DNases expressed by several common taxa identified in endodontic infections including Porphyromonas spp., Prevotella spp., Fusobacterium spp., or T. forsythia. In addition, it is worth recalling that a healthy pulp is devoid of any resident microbiota prior to bacterial colonization, which contrasts with more commonly sampled oral sites such as saliva or plaque. Therefore, even if DNA from dead bacteria was detected, it is highly unlikely that such results would lack significance with regard to disease causation as any bacteria identified may contribute to the ecology of the microbiota.

Other sources of variations between studies may be related to the difficulty of reliably sampling the complex anatomy of root canals, typically displaying numerous ramifications, e.g., in the apical portion, which shelters bacterial biofilms responsible for disease persistency and are therefore of prime clinical interest (Figure 2). In this regard, the paper point sampling method (11/18 studies) is commonly criticized for its inability to reach the apical portion and its ramifications, thereby somewhat limiting the sampling to planktonic bacteria from the main
root canal. In addition, this method only provides an average picture of the taxa present, without permitting to distinguish between the coronal or apical sections of the root canal. In contrast, the cryo-pulverization alternative (6/18 studies) permits the selective analysis of the microbiota associated with the apical portion of the root, and most importantly to entirely sample biofilm communities enmeshed in radicular irregularities. One important disadvantage of cryo-pulverization remains that it most often requires the extraction of the tooth and is therefore preferred in heavily infected and recalcitrant cases (Figure 2).

It is also inevitable that variations exist between studies using complex NGS workflows, in which potential biases may occur during the DNA extraction, PCR amplification, sequencing, or bioinformatic analysis pipeline. Different DNA extraction protocols may demonstrate varying efficiencies in lysing different bacterial cells, thereby preferentially yielding higher DNA concentrations from particular taxa over others. Different taxa also bear varying numbers of 16S rRNA gene copies, therefore affecting the total yield of amplicons obtained. The choice of the 16S rRNA gene variable regions to be amplified is another potential variation factor, as not all regions display comparable sequence diversity and ability to distinguish different taxa. This is particularly crucial when analyzing short amplicons such as the ones generated by Illumina technologies (paired-end reads up to 2 × 300 bp). As an example, the included studies mostly spanned regions V1–V2 and V3–V4 (Figure 3). Regions V1–V2, however, were suggested to yield less reproducible results and to potentially underestimate certain taxa such as Bifidobacterium. Finally, raw sequencing data require several bioinformatic steps to render them interpretable. Reads are typically quality-filtered, paired, clustered, assigned, decontaminated, and normalized. Different approaches to these steps may result in additional variations. It is well recognized for instance that differences in taxonomic assignment may easily stem from different clustering thresholds or different 16S rRNA databases searched.

Because of this propensity to variations and high sensitivity, carrying out definitive NGS experiments demands great care and various sets of controls. Negative extraction controls (NECs) serve to assess the presence of contaminating sequences known to originate from reagents, kits, disposables, or manipulations. These are typically recommended when sampling sites with low bacterial DNA, in which the amplicons preparation and sequencing were shown to return sequences mainly derived from contamination. NECs are typically generated by using a blank paper point or instrument that undergoes the full extraction and sequencing protocol. Among the studies included, only three of 18 reported to have run NECs in parallel with their root canal samples. In addition to NECs, negative controls serve to assess the contamination background specific to the sampling site. Herein, 12 of 18 studies have included negative controls mainly taken from endodontic access cavities or external root surfaces after disinfection, to verify the relative “sterility” of the site prior to sampling. Among these 12 studies, four used vital teeth as negative controls, which could also be regarded as biological/healthy controls. However, ten of 12 studies have actually sequenced their negative controls. A risk of bias assessment was conducted for the outcome of interest considered in the current systematic review, i.e., the microbiota composition in PAPs, SAPs, and AAs (Table 3). Among the 18 articles included, 12 were evaluated as “low risk” (unlikely to affect the outcome of interest), two were considered to raise some “concerns” (unpredictable effect on the outcome of interest), and four were judged as “high risk” (likely to affect the outcome of interest) (Table 3).

Prior to the advent of NGS technologies, the microbiota of infected roots was more classically investigated by means of culture and close-ended molecular methods, such as PCR (and derivatives), DNA hybridization assays, or fluorescent in situ hybridization (FISH). These more classical approaches permitted to catalogue a set of 20–30 bacterial species presumed to play an important role in the pathogenesis of PAP and SAP lesions. In comparison, the NGS studies herein, neither limited to these strains that can be cultured in the laboratory nor...
Table 3. Risk of bias table for the outcome of interest addressed in the current systematic review. NTR; nothing to report.

| Study                | Li et al. (2010) | Santos et al. (2011) | Siqueira et al. (2011) | Hsiao et al. (2012) | Özok et al. (2012) | Hong et al. (2013) |
|----------------------|-----------------|----------------------|------------------------|---------------------|-------------------|-------------------|
| Suspected bias domain| 1) Bias in selection of participants | Bias due to sampling methodology | NTR | NTR | NTR | NTR |
|                      | 2) Bias in selective reporting | | | | | |
| Judgement            | High risk       | High risk            | Low risk               | Low risk            | Low risk          | Low risk          |
| Support for judgement| 1) Indiscriminate inclusion of six PAP and one SAP samples. Whereas discriminating between PAPs and SAPs was not the aim of the authors, it may affect the outcome of interest of the current systematic review. | Comparison between PAPs and AAAs was conducted from different sampling sites. Whereas PAPs were sampled from root canals, AAAs were sampled extra-radically by pus aspiration. Only AA samples are therefore considered a potential bias to the current systematic review. | NTR | NTR | NTR | NTR |
|                      | 2) Unspecified OTU clustering cutoff | | | | | |

| Study                | Anderson et al. (2013) | Vengerfeldt et al. (2014) | Tzanetakis et al. (2015) | Gomes et al. (2015) | Siqueira et al. (2016) | Persoon et al. (2017) |
|----------------------|------------------------|--------------------------|------------------------|---------------------|------------------------|----------------------|
| Suspected bias domain| NTR                    | Bias in selective reporting | Bias in selective reporting | Bias in selection of participants | Bias in selection of participants | NTR |
| Judgement            | Low risk               | Low risk                 | Low risk               | Some concerns        | Some concerns          | NTR |
| Support for judgement| NTR                    | The authors did not report the relative abundance of bacterial composition at the genera level, although they mention some genera and species in the result and discussion sections. Additionally, the OTU clustering cutoff was not reported. | Unspecified OTU clustering cutoff | The authors aimed at investigating the root microbiota associated with endo-peri lesions, i.e., infections of the periodontium that transfer to the root canals and/or inversely. However, the criteria set may not stringently select this population of interest (see Discussion section). | In this study, the roots were collected by apical surgery. Bias concern 1) Root resection during surgery is guided by bio-medical imperatives rather than microbial sampling. Bias concern 2) Teeth with SAP typically considered eligible for surgery have usually undergone several endodontic treatments, as described by the authors (Material & Methods, Case description). The potential bias induced on this systematic review is therefore judged unpredictable. | NTR |

(Continued)
Table 3. Continued.

| Study                                | Keskin et al. (2017) | Sanchez-Sanhueza et al. (2018) | Iriboz et al. (2018) | Zandi et al. (2018) | Bouillaguet et al. (2018) | Qian et al. (2019) |
|--------------------------------------|----------------------|---------------------------------|---------------------|----------------------|-------------------------|-------------------|
| Suspected bias domain                | NTR                  | NTR                             | 1) Bias in selective reporting | 2) Bias due to sampling methodology | NTR                     | NTR               |
| Bias due to sampling methodology    | High risk            | Low risk                        | Comment: disclosure of conflict of interest, one of the authors of the current systematic review was also involved in this study |                      |                         |                   |
| Bias due to sampling methodology    | Low risk             | Low risk                        |                      |                      |                         |                   |
| Support for judgement                | NTR                  | NTR                             |                      |                      |                         |                   |
| Judgement                            | Low risk             | Low risk                        |                      |                      |                         |                   |
| Comment: the authors did not report any taxonomic assignment below the Family level for none of the 86 OTUs identified. |                      |                      |                      |                      |                         |                   |
| Bias concern 1) Only DNA from dead cells was hydrolyzed prior to DNA extraction and amplification. |                      |                      |                      |                      |                         |                   |
| Bias concern 2) This is the only report that performed a DNA from live bacteria only effect on the outcome of interest is unpredictable. |                      |                      |                      |                      |                         |                   |

When looking into particular communities’ composition, NGS studies most often corroborated previous findings. For instance, NGS reports largely confirmed the high abundances of previously identified species such as *Fusobacterium nucleatum*, *Porphyromonas spp.*, or *Prevotella* spp. in PAPs (Table 2). Also, NGS findings confirmed the involvement in PAPs of several strictly anaerobic Firmicutes such as *Pseudoramibacter lacolyticus*, *Dialister pneumosintes*, or *Propionibacterium micras.* Together, these strictly anaerobic, proteolytic species, and sometimes saccharolytic (*Dialister* spp. and some *Porphyromonas* species), appeared predominant in PAPs. In other instances, NGS studies permitted to refine previously reported results. For instance, representatives of the phylum Synergistetes were detected in infected root canals by PCR and Sanger sequencing approaches in the early 2000s. The taxa, however, were yet uncultured and only designated as clones BA121 and W090. Further studies that relied on FISH confirmed the presence of Synergistetes taxa in close proximity to apical lesions, more abundantly in SAPs, but were unable to assign lower taxonomy. Interestingly, clones BA121 and W090 respectively belong to the genera currently named *Pyramidobacter* and *Fretibacterium*, both of which have been abundantly detected in SAPs by several NGS reports included here (Table 2).

In other cases, previous results where rather nuanced by NGS findings. For instance, the species *E. faecalis* was classically the most frequently retrieved bacterium from SAPs-associated root canals, with prevalence values reaching up to 90%, but was rarely detected in PAPs. Although NGS results somewhat confirm that *E. faecalis* is preferentially detected in SAPs, the species was detected in significantly lower frequencies; four of 10 SAPs (40%) in Siqueira et al., two of ten SAPs (20%) in Zandi et al., and seven of 22 SAPs (32%) in Bouillaguet et al. (Table 2). Interestingly, despite lower prevalence values, the species could reach fairly high average abundances; 14% in Zandi et al. and 20% in Bouillaguet et al., indicating that some canals bear remarkably high loads of *E. faecalis*. In this line, a hierarchical cluster analysis performed by Bouillaguet et al. showed a clear clustering of SAPs according to their content of *E. faecalis* whereas some SAPs appeared devoid of the species, others exhibited abundances above 90%. There is evidence showing that *E. faecalis* is more commonly retrieved from teeth treated in multiple visits or temporarily left open. This may suggest that the species is a secondary opportunistic colonizer not necessarily part of the microbiota present in PAPs prior to treatment. Although the term SAP commonly refers to treated root canals associated with apical lesions, specialists further distinguish persistent infections caused by bacteria present before the initial treatment, from secondary infections caused by bacteria invading the root canal following treatment. Whereas this dichotomy is often clinically impossible, it might be reflected into these canals heavily colonized by *E. faecalis*. 

to a range of pre-defined species, identified on average 400 OTUs (species-level taxa) in infected root canals (PAPs, SAPs, and AAs; Table 1). One important observation that may be derived from this high richness, is that there are virtually no species unequivocally specific to an infection type. In other words, the same taxa tend to be detected in every endodontic infections, yet with different relative abundances.
Taken together, NGS technologies appeared as a tool of choice to detect the highly diverse microbiotas associated with PAPs, SAPs, and AAs. Findings from these studies provided evidence of compositionally unspecific, yet differently abundant bacterial communities in each infection type. Synthesis of these findings confirmed the presence of previously identified taxa in each infection type, and further expanded the list to a myriad of low abundant taxa with yet-poorly understood clinical relevance. From an ecological standpoint, it has been demonstrated that all members of a mixed consortium are important to detect, as they might serve as keystone species necessary to establish the virulence of the entire community.[104,105] These low-abundant species might have been dominant in the past or have the potential to become dominant in the future in response to environmental shifts that favor their growth.[106] Beyond community surveys, further ecological insights will surely originate from whole-genome sequencing (WGS) studies that circumvent the need for targeted studies.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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