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**16SpeB: Towards defining bacterial species boundaries by intra-species gene sequence identity**

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**Running head:** 16S bacterial species boundary

**Key words:** 16S rRNA gene; 16S amplicon sequencing; high-throughput sequencing

20 **ABSTRACT**

21 **Summary:** 16SpeB (16S rRNA-based Species Boundary) is a package of Perl programs that  
22 evaluates total sequence variation of a bacterial species at the levels of the whole 16S rRNA  
23 sequences or single hypervariable (V) regions, using publicly-available sequences. The 16SpeB  
24 pipelines filter sequences from duplicated strains and of low quality, extracts a V region of  
25 interest using general primer sequences, and calculates sequence percentage identity (%ID)  
26 through all possible pairwise alignments.

27 **Results:** The minimum %ID of 16S rRNA gene sequences for 15 clinically-important bacterial  
28 species, as determined by 16SpeB, ranged from 82.6% to 99.8%. The relationship between  
29 minimum %ID of V2/V6 regions and full-gene sequences varied among species, indicating that  
30 %ID species limits should be resolved independently for each region of the 16S rRNA gene and  
31 bacterial species.

32 **Availability:** 16SpeB and user manual are freely available for download from:

33 <https://github.com/pnpnpn/16SpeB>. A video tutorial is available at:

34 <https://youtu.be/Vd6YmMhyBiA>

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36 **Supplementary information:** Supplementary data are available at Bioinformatics online.

## 37 **1 INTRODUCTION**

38 Fueled by recent advance in next-generation sequencing (NGS), nucleic-acid-based  
39 identification of microbes from clinical and environmental samples is an emerging area of  
40 scientific interests (Kress, et al., 2015; Shokralla, et al., 2012; van Dijk, et al., 2014; Wilson, et  
41 al., 2014). For bacteria, gene markers such as the 16S ribosomal RNA (rRNA) gene are  
42 commonly used to profile communities that encompass both cultured and uncultured species. An  
43 enduring challenge is to assign taxonomy to these marker gene sequences, especially, to assess  
44 the confidence a particular sequence read fits into its designated taxonomic rank based on  
45 percentage identity (%ID); and be able to discriminate rare or novel taxa from taxa likely arisen  
46 from sequencing errors.

47 Over time, scientists have attempted to find a unifying threshold to define bacterial  
48 species boundary from their gene sequences. For example, a 97% sequence identity (%ID) of the  
49 full length 16S rRNA gene has been put forward as the cut-off value to define species  
50 (Drancourt, et al., 2004; Drancourt and Raoult, 2005; Ueda, et al., 1999), but the criterion has  
51 been vigorously challenged (Clarridge, 2004; Janda and Abbott, 2007; Petti, 2007; Rossi-  
52 Tamisier, et al., 2015). Compounding the uncertainty about using a fixed %ID threshold for  
53 species identification, it is becoming a common trend to sequence shorter but varied reads (<400  
54 bp) of single hypervariable (V) regions, such as the V2 or V6 of the 16S rRNA gene (Bowen, et  
55 al., 2011; De Filippo, et al., 2010; Guss, et al., 2011; Kirchman, et al., 2010; Ravussin, et al.,  
56 2011; Werner, et al., 2012; Wu, et al., 2011).

57 To address some of the caveats associated with 16S rRNA gene profiling, especially to  
58 facilitate more confident taxonomy assignment, we proposed that the 16S rRNA %ID variation  
59 from known sequences shall be determined and used to guide the boundary of bacteria species-

60 to-species. We thus develop 16SpeB (16S rRNA-based Species Boundary). 16SpeB is an  
61 analytical tool designed to identify the range of 16S %ID encompassed by individual bacterial  
62 species based on known 16S rRNA gene sequence variation. Our goal is to promote accurate  
63 taxonomic identification of bacteria in both (near)-full 16S sequences and short reads obtained  
64 by 454, Illumina or other next-generation sequencing platforms.

65

## 66 **2 USAGE**

67 16S rRNA sequences from three 16S rRNA databases can be downloaded from  
68 *Greengenes* (DeSantis, et al., 2006) *Ribosomal Database Project* (Cole, et al., 2007) and *Silva*  
69 (Pruesse, et al., 2007). 16SpeB allows users to trim the (near)-full 16S rRNA sequences to their  
70 preferred length. It can also extract the sequences of the V2 and V6 regions, which are widely  
71 used in 454 sequencing studies, by reference to the general primer sets 27F-338R and 784F-  
72 1061R, respectively. Sequences that fail to satisfy the two following conditions are removed: (1)  
73 <2 bp mismatches with the general 16S primers (i.e. conserved regions of the 16S gene), and (2)  
74 relative coordinates of matched primers are within +/- 50 bp from the relative coordinates of the  
75 literature. The V2 region is trimmed to 270 bp upstream of the 338R primer. 16SpeB conducts all  
76 possible pairwise sequence comparisons by aligning all pairwise sequences using Needleman-  
77 Wunsch alignment algorithm with match/mismatch score of 1/-2 and affine gap penalty  
78 open/extension of -5/-2. The minimum and 95% quantile %ID are computed for each species,  
79 providing a measure of the total known sequence variation that defines the species.

80

## 81 **3 APPLICATION OF 16SpeB**

82 16SpeB was initially developed to identify species limits of *Acetobacter* and *Lactobacillus* in a  
83 pyrosequencing analysis of the gut microbiota of *Drosophila melanogaster* (Wong, et al., 2011).  
84 Here we extend the application of 16SpeB to determine the %ID of (near-)full 16S rRNA genes  
85 that defines the species boundary of 15 clinically-important bacterial species (listed in  
86 Supplementary Data Set 1); and to determine the %ID of the V2 and V6 regions widely used in  
87 pyrosequencing studies that correlate with this species boundary. The 15 bacterial species were  
88 selected on the criteria that a broad range of publicly-available sequences (3 to 454) and  
89 phylogenetic diversity (including representatives of Actinobacteria, Bacteroidetes, Chlamydiae,  
90 Firmicutes and Proteobacteria) were represented. In total, 1,296 sequences were analyzed. The  
91 minimum %ID of (near-) full 16S sequences varied from 99.8% (*Neisseria gonorrhoeae*) to  
92 82.6% (*Staphylococcus aureus*) (Table 1). Just two (13%) of the 15 species had minimum %ID  
93 close to predicted 97% threshold for species boundary (*Neisseria meningitidis* 97.0%, and  
94 *Listeria monocytogenes* 97.1%); and 11 (73%) species deviated from 97% by more than one  
95 percentage point. Values of the 95% quantile are provided in Table 1 and may prove to be more  
96 useful than minimum %ID for some species, e.g. *Staphylococcus aureus*, where the minimum  
97 %ID is suspected to be artefactually low (possibly through mis-identification).

98 As anticipated, the minimum %ID of both the V2 and V6 regions varied positively with  
99 %minimum ID of the (near-) full sequence of the 16S genes (Supplementary Figure 1). The  
100 relationships were not, however, tight indicating that the rates of sequence evolution of  
101 individual V regions are not closely correlated to each other or to other regions of the 16S gene.  
102 The implication is that, just as the 97% threshold is not a reliable index of the taxonomic species  
103 limit, so there is no simple linear relationship linking the minimum %ID of the V2 or V6  
104 sequences to the (near-) full 16S sequence across multiple bacterial species.

105 We conclude that the %ID species limits should be resolved independently for each region of  
106 the 16S rRNA gene and each bacterial species. Therefore, 16SpeB can serve as an important tool  
107 that facilitates accurate taxonomic identification and proper interpretation of 16S rRNA gene  
108 pyrosequencing data.

109

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116 TABLE 1. The minimum and 95% quantile %ID of the (near-)full 16S rRNA gene, and the V2 and V6 regions of the 15 clinically-  
 117 important bacteria

Species	Number of sequences (pairs)	Minimum %ID			95% quantile %ID		
		(near)-full 16S	V2	V6	(near)-full 16S	V2	V6
<i>Bacteroides fragilis</i>	345 (59340)	0.928	0.899	0.928	0.978	0.959	0.973
<i>Clostridium bifermentans</i>	58 (1653)	0.926	0.928	0.787	0.967	0.967	0.893
<i>Chlamydia trachomati</i>	15 (105)	0.950	0.921	0.954	0.958	0.942	0.959
<i>Corynebacterium diphtheriae</i>	10 (45)	0.942	0.920	0.912	0.946	0.934	0.918
<i>Haemophilus influenzae</i>	92 (4186)	0.901	0.831	0.891	0.951	0.925	0.907
<i>Helicobacter pylori</i>	59 (1711)	0.949	0.895	0.939	0.977	0.960	0.961
<i>Listeria monocytogenes</i>	26 (325)	0.971	0.939	0.966	0.974	0.953	0.969
<i>Mycobacterium leprae</i>	4 (6)	0.984	0.967	0.992	0.984	0.967	0.992
<i>Mycobacterium tuberculosis</i>	10 (45)	0.984	0.982	0.988	0.989	0.985	0.992
<i>Mycoplasma hominis</i>	6 (15)	0.899	0.949	0.681	0.899	0.949	0.681
<i>Neisseria gonorrhoeae</i>	3 (3)	0.998	1.000	1.000	0.999	1.000	1.000
<i>Neisseria meningitidis</i>	133 (8778)	0.970	0.927	0.962	0.990	0.978	0.981
<i>Staphylococcus aureus</i>	454 (102831)	0.826	0.604	0.843	0.980	0.981	0.973
<i>Streptococcus pneumoniae</i>	47 (1081)	0.980	0.938	0.977	0.986	0.963	0.985
<i>Yersinia pestis</i>	34 (561)	0.979	0.960	0.966	0.986	0.967	0.977

118 SUPPLEMENTARY DATA SET 1. List of 16S rRNA sequences used in the study  
119 SUPPLEMENTARY FIGURE 1. Relationship between a) minimum and b) 95% quantile %ID of V2/V6  
120 region and (near)-full 16S rRNA gene sequence across the 15 bacterial species used in this study. (V2  
121 region: black, solid squares; V6 region: grey, open circles).

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