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Short communication

Identification of *Brachyspira* species by *cpn60* universal target sequencing is superior to NADH oxidase gene sequencing

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ABSTRACT

The pig colon is the habitat of diverse *Brachyspira* species, of which only a few are of clinical importance. Methods for identification have shifted from phenotypic to molecular testing over the last two decades. Following the emergence of *B. hampsonii* it became evident that relying on species-specific PCRs carries the risk of overlooking important new species. Consequently, sequencing was proposed as an unbiased alternative for identification of isolates. So far, the main target for identification across species has been the NADH oxidase gene (*nox*). However, multiple copies of this gene in the genome and potential lateral gene transfer reduce confidence when using this gene. This study compared identification and phylogentic relationship inferred from *nox* sequencing to that inferred from sequencing of the *cpn60* universal target using a collection of 168 isolates from different *Brachyspira* species. The majority of isolates had an identical identification with both methods. There were a few outliers in the trees with uncertain assignment to a species by BLAST analysis. A few major discrepancies pertained to the pathogenic species *B. hampsonii* (2), *B. pilosicoli* (1) and *B. suanatina* (1). Weakly haemolytic variants of *B. hyodysenteriae* were assigned to the correct species by both methods. Some of the isolates identified as *B. hampsonii* also had a weakly haemolytic phenotype.

1. Introduction

Over the last 20 years more and more spirochaete species within the genus *Brachyspira* (*B.*) have been described. Although in many bacteria 16S rRNA gene sequencing is the reference method for species identification, this does not readily discriminate between *Brachyspira* species (Stanton et al., 1996). With this genus, partial sequencing of the NADH oxidase gene (*nox*) is widely used for this purpose (Atyeo et al., 1999). However, the diagnostic reliability of this locus has been questioned, especially in the light of the potential transduction of the *nox* gene by phage VSH-1 (Humphrey et al., 1997) and recently observed discrepancies between phenotypic (weakly versus strongly haemolytic) and genotypic identification in *B. hyodysenteriae* (Card et al., 2019).

Chaperonins are a diverse family of molecular chaperones that are present almost in all eubacteria and archaea. Chaperonin sequences are useful for phylogenetic studies since a 549–567 bp segment of the *cpn60* coding region, the "universal target" (UT), can be amplified with universal PCR primers. Usually it is represented by a single copy in the bacterial genome. Sequence variation extends uniformly throughout the coding region and neither the gene itself nor its transcript shows a stable secondary structure that would impact the chances for sequence variation (Hill et al., 2004). It has been demonstrated that the *cpn60* UT region generally provides more discriminating and phylogenetically informative data than the 16S rRNA gene target (Verbeke et al., 2011; Links et al., 2012). In this study, field isolates of different *Brachyspira* species previously identified by partial *nox* sequencing were compared based on the *cpn60*UT sequence. Dendrograms for each locus were drawn, and isolate clustering based on each gene was compared.

2. Material and methods

Sequences from 168 *Brachyspira* strains and isolates were compared, including published sequences from type and reference strains (supplemental table 1 and 2). The sequences used included those from 5 isolates from Lesser Snow Geese (*Chen caerulescenc caerulescens*) (SHI-067, SHI-068, CH-003, CH-014 and KL-207), *B. pilosicoli* B2904 isolated

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from a chicken, *B. pilosicoli* WesB isolated from a human child, *B. aalborgi* ACTC43994 from a human patient, with all the other isolates being from pigs from Germany, The Netherlands, Australia and Canada. *Nox* sequencing with genus-specific primers and *cpn60*UT sequencing were done as previously published (Hill et al., 2006; Rohde et al., 2002). Trimmed sequences were analyzed using MEGA version 7 (Kumar et al., 2016) implementing ClustalW for alignment.

Phylogenetic trees were generated using FastTree (Price et al., 2010). The reliability of each split in the tree was calculated by FastTree using the Shimodaira-Hasegawa test with 1000 resamples. Tree images were generated using the interactive Tree Of Life (iTOL) online tool https://itol.embl.de/ (Letunic and Bork, 2016).

Nox sequences of tree outliers also were analyzed using Megablast with default settings and the database Nucleotide collection nr/nt (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Similarly, *cpn60*UT sequences were analyzed using FASTA to query a database of *cpn60* nucleotide sequences (http://www.cpndb.ca/).

Additionally, species-specific PCRs for *B. suanatina* (*rpo C*) and *B. pilosicoli* (16S rRNA gene) were set up as previously published (Fellstrom et al., 1997; La et al., 2016a) or specifically designed to target *tlyA* of *B. hampsonii* (tlyAhamp-F: AAAGCCTTTATAGAATTTGG TATATC; tlyAhamp-R: AATAGGGGCTATTTTTGATATTGAT; size of the product 288 bp).

Haemolysis of the *B. hyodysenteriae*, *B. suanatina* and *B. hampsonii* isolates was assessed on Tryptic Soy Blood Agar with 10% bovine blood, including evaluation of the ring phenomenon with a defined zone of enhanced translucency for strongly haemolytic isolates where the agar was sliced in the spirochaete growth. For some Canadian sequences haemolysis was not recorded and no isolates were stored for retrospective evaluation of haemolysis (s. supplemental table 2).

3. Results

Species within the genus *Brachyspira* were well delineated. All isolates identified as *B. hyodysenteriae* by *nox* sequencing (Fig. 1) also clustered together with *B. hyodysenteriae* reference and type strain sequences in the *cpn60* tree (Fig. 2). In the NADH oxidase gene tree, *B. hyodysenteriae* isolates showed very little diversity, except for two weakly haemolytic isolates from Australia (TL_wh1 and TL_wh2), which were grouped separately. However, this separation was only supported by a bootstrap value of 0.6. In BLAST analysis the *nox* gene sequence of these isolates showed 98.8% identity with the *nox* gene of the type strain *B. hyodysenteriae* B78 (Table 1). The *B. hyodysenteriae* cluster in the *cpn60* tree fell into several sub-clusters that were supported by bootstrap values of 0.7-0.9. Interestingly, cluster I-III only incorporated weakly haemolytic isolates. Isolates in cluster I were from different but epidemiologically related German farms. Cluster II contained related isolates and isolates without known relationship from Germany and The Netherlands, respectively. Two German weakly haemolytic isolates without known relationship constituted cluster III. The two Australian weakly haemolytic isolates (TL_wh1 and TL_wh2) clustered together with strongly heamolytic isolates from Germany, Canada, Australia and the type strain B78.

The *B. hampsonii* cluster encompassed weakly and strongly hemolytic isolates in both trees. The weakly haemolytic isolates were not separated from the strongly haemolytic isolates either in the NADH oxidase gene dendrogram or in the *cpn60* tree. Weakly haemolytic *B. hampsonii* field isolates from German pigs were positive in a PCR designed to detect the *tlyA* gene of this *Brachyspira* species. Two weakly haemolytic isolates from geese (SHI-067 and SHI-068) identified as *B. hampsonii* by *nox* gene sequencing (Fig. 1 and Table 1) clustered in the *B. murdochii-B. innocens*-complex with *cpn60*UT sequencing (Fig. 2).

One weakly hemolytic isolate (2191-1x/14) with 97.8% identity to *B. suanatina* type strain AN4859-03 in the *nox* gene (Table 1) grouped more closely with *B. intermedia* in the *cpn60*UT tree (Fig. 2), while two strongly haemolytic field isolates (1972/15 and 755-3x/17) clustered closely with the *B. suanatina* type strain AN4859-03 in both trees. In a *B. suanatina* species-specific PCR targeting the *rpoC* gene the type strain and the strongly haemolytic isolates were positive, whereas the weakly haemolytic isolate did not amplify.

All except three *B. pilosicoli* isolates grouped into a common cluster with both sequencing methods. Two of these isolates (6761-13x/18 and T079) distantly grouped with the *B. pilosicoli* cluster by *nox* sequencing (Fig. 1) and were found in the *B. innocens-B. murdochii*-cluster of the *cpn*60UT tree (Fig. 2). However, only FASTA analysis of the *cpn60*UT of isolate 6761-13x/18 resulted in an acceptable identification with 99.1% similarity to the *cpn60*UT of *B. innocens* B256 (Table1). Notably, 6761-13x/18 also was negative in a 16S rRNA gene-based *B. pilosicoli* species-specific PCR. Finally, one *B. murdochii* isolate (T062) by *nox* sequencing is a *B. pilosicoli* by *cpn60* with similarities to the respective type strains of > 99% (Table 1).

B. murdochii and *B. innocens* were separated from the other *Brachyspira* species in both trees (Figs. 1 and 2). There were several mismatches between *nox* and *cpn60* sequencing within this group of isolates and also with other *Brachyspira* species. Two *B. murdochii* isolates (SHI-067 and SHI-068) by *cpn60* sequencing were grouped with the *B. hampsonii* isolates in the *nox* tree. One *B. innocens* isolate (6761-13x/19) and one *B. murdochii* isolate (T079) were clustered with *B. pilosicoli* by *nox* sequencing. One published sequence (D13-03603F2) designated as belonging to *B. murdochii* by whole-genome and *nox* sequencing as well as several field isolates grouped in a shared sub-cluster of the *cpn60* tree that seems closer to the sub-cluster with the *B. innocens* type strain than to the sub-cluster with the *B. murdochii* type strain. Lastly, two isolates (T057 and T103) located in the *B. intermedia* cluster of the *nox* tree (Fig. 1) were designated as *B. murdochii* by *cpn60* sequencing (Table 1).

4. Discussion

The pig colon is the habitat of diverse *Brachyspira* species, only some of which are of clinical importance. Methods for identification have shifted from phenotypic to molecular based testing over the last two decades. Following the emergence of *B. hampsonii* it became evident that using species-specific PCRs carries the risk of overlooking such new and important species (Burrough, 2017; Rohde et al., 2014). Consequently, sequencing is proposed as an unbiased alternative for identification of isolates. Unlike many other bacteria, sequencing of the 16S rRNA gene is not sufficiently discriminatory for identification of *Brachypira* species (Stanton et al., 1996). In such cases it is well accepted in molecular taxonomy to use protein-encoding genes instead that fulfill certain criteria and thus also allow for some degree of phylogenetic reconstruction in closely related bacteria (Harayama and Kasai, 2006). In *Brachyspira,* partial sequencing of the *nox* gene using genus specific primers is widely used for identification (Atyeo et al., 1999; Johnson et al., 2018; Patterson et al., 2013). However, the *nox* gene exists in two copies in the *B. hyodysenteriae* genome and is potentially prone to horizontal gene transfer (Bellgard et al., 2009; Humphrey et al., 1997). On the other hand, the *cpn60* gene exists in all bacterial taxa, usually as a single copy, and with uniform sequence variations throughout the gene (Hill et al., 2004). Universal primers for amplification and sequencing of a core region, called the Universal Target (UT), and corresponding to nucleotides 274–828 of the *E. coli cpn60* sequence are published (Hill et al., 2006). A publically accessible database of chaperonin sequences is available (http://www.cpndb.ca/).

Overall, identification of all *Brachyspira* species was identical with both *nox* and *cpn60*UT sequencing, with a few notable exceptions. These exceptions mainly pertained to isolates that were assigned to the *B. murdochii-B. innocens*-cluster in the *cpn60* tree but were grouping with or close to *B. hampsonii, B. pilosicoli* or *B. intermedia* in the *nox* gene tree. Species-specific PCRs for the pathogenic species indicate that the *cpn60*UT sequencing result is more reliable than *nox* sequencing. This is also true for one *rpoC*-PCR negative isolate classified as *B. intermedia* by

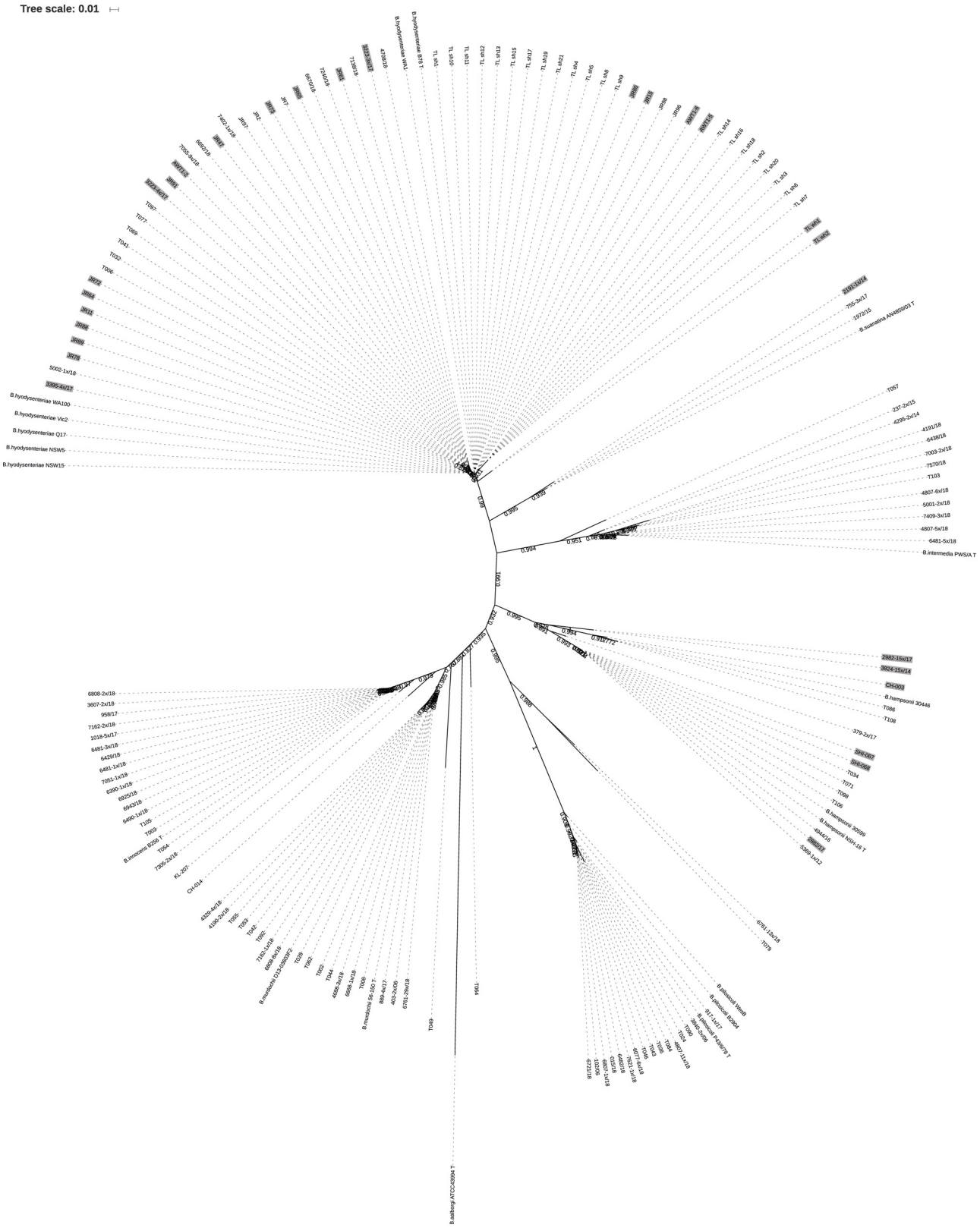

Fig. 1. Relationship of 168 *Brachyspira* isolates inferred from NADH oxidase gene sequences. Scale: base substitutions per site. Only bootstrap values > 0.70 are indicated (1000 iterations). Weakly haemolytic isolates of *B. hyodysenteriae*, *B. hampsonii* and *B. suanatina* are indicated by light grey shading.

*cpn60*UT sequencing but as *B. suanatina* by *nox* sequencing.

Identification of weakly haemolytic isolates as *B. hyodysenteriae* has only recently been confirmed by whole-genome sequencing (Card et al., 2019), and representatives of that study were included in our analysis. For *B. hyodysenteriae*, *cpn60*UT sequencing provided higher resolution of isolates. It is interesting to note that weakly haemolytic *B. hyodysenteriae* isolates from Germany and The Netherlands clustered in three clusters separate from the strongly haemolytic isolates. Two of these

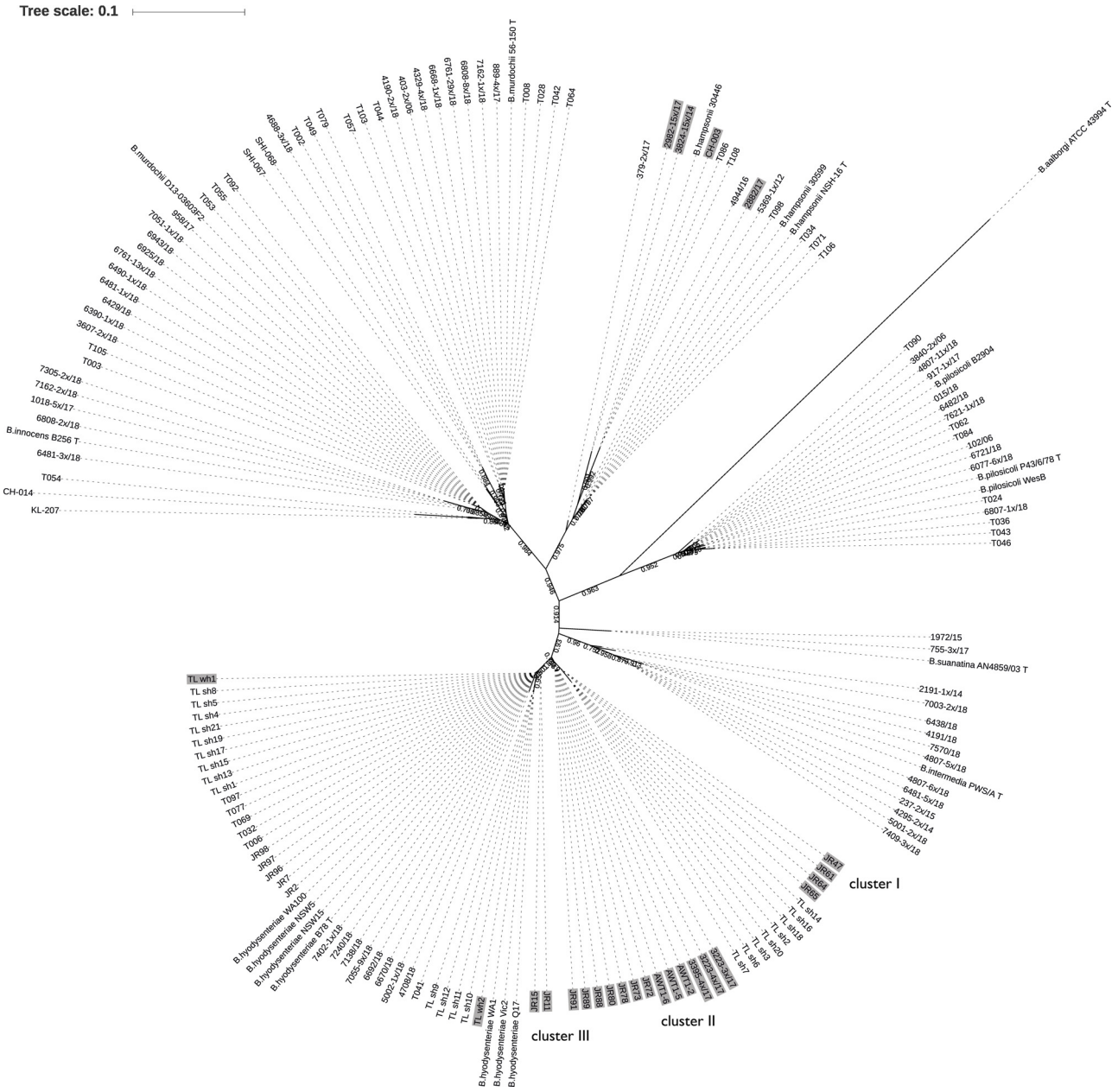

Fig. 2. Relationship of 168 *Brachyspira* isolates inferred from *cpn60*UT sequences. Scale: base substitutions per site. Only bootstrap values > 0.70 are indicated (1000 iterations). Weakly haemolytic isolates of *B. hyodysenteriae* and *B. hampsonii* are indicated by light grey shading.

isolates clustering together (cluster III: JR11 and JR15) differed from all other weakly haemolytic isolates by the fact that they have an insertion in the proposed *hlyA* promoter region (La et al., 2016b). Some of the weakly haemolytic isolates in cluster II have known epidemiological relationships and some do not. Consequently, it is difficult to say whether the separation into these clusters may only reflect an epidemiological relation or the beginning of phylogenetic diversity. Isolate JR72 was classified as weakly haemolytic by growth on TSA and ring phenomenon, and grouped with the weakly haemolytic isolates in the *cpn60* tree. However, titration of haemolysis revealed that its haemolytic capacity may be growth (phase) dependent (Card et al., 2019). It also shared other characteristics with strongly haemolytic as well as with weakly haemolytic isolates (Card et al., 2019). The two Australian weakly haemolytic *B. hyodysenteriae* clustered with strongly haemolytic isolates. Since the Australian pig population has been segregated from the rest of the world for about 35 years when live pig imports were banned, the weakly haemolytic phenotype of *B. hyodysenteriae* seems to have developed independently on different continents.

Interestingly, a few isolates grouping in the several *B. hampsonii* subclusters of this diverse species exhibit a weakly haemolytic phenotype which is not in accordance with the species description (Mirajkar et al., 2016). It is to be expected that these isolates represent a phenotypic variant similar to the weakly haemolytic *B. hyodysenteriae* and it would be interesting to compare whole-genome sequences of both haemolysis types as has been done for *B. hyodysenteriae*. In a broader genotypic study, one of our weakly haemolytic isolates (38241-5x/14) was assigned to genetic group III of this species whereas the strongly haemolytic isolate 5369-1x/12 belonged to genetic group I (Mirajkar et al., 2015). Two pigs on different farms from which strongly haemolytic isolates were cultured showed mild (isolate 379-2x/17) or moderate diarrhoea (isolate 5369-1x/12), respectively, and pigs from another farm with a strongly haemolytic isolate had bloody scours (isolate 4944/16). Two weakly haemolytic isolates (2982-15x/17 and 3824-15x/14) were from surveillance samples. One of these farms

Table 1
Results of BLAST analysis of *nox* sequences[a] and FASTA analysis of *cpn60*UT sequences[b] for isolates that are outliers in the respective trees.

lab ID	*nox* bp[c]	*nox* identity %[d]	strain	*cpn60*UT identity %[d]	strain	*cpn60*UT identity %[d]	strain
237-2x/15	876	98.5	*B. intermedia* PWS/A^T	98.9	*B. intermedia* PWS/A^T		
2191-1x/14	896	97.8	*B. suanatina* AN4859/03^T	98.4	*B. intermedia* PWS/A^T		
379-2x/17	906	97,6	*B. hampsonii* NSH-16^T	98.4	*B. hampsonii* 30599		
4295-2x/14	918	98.5	*B. intermedia* PWS/A^T	98.9	*B. intermedia* PWS/A^T		
6761-13x/18	899	93.2	*B. innocens* B256^T	99.1	*B. innocens* B256^T		
7003-2x/18	901	97.5	*B. intermedia* PWS/A^T	98.0	*B. intermedia* PWS/A^T		
CH-104	710	97.0	*B. murdochii* 56-150^T	97.1	*B. innocens* B256^T	96.9	*B. innocens* B256^T
JR11	827	100	*B. hyodysenteriae* B78^T	99.8	*B. hyodysenteriae* B78^T		
JR15	881	100	*B. hyodysenteriae* B78^T	99.8	*B. hyodysenteriae* B78^T		
KL-207	710	97.8	*B. innocens* B256^T	98.0	*B. innocens* B256^T	97.8	*B. murdochii* 56-150^T
SHI-067	710	100.0	*B. hampsonii* NSH-16^T	97.8	*B. murdochii* 56-150^T	97.8	*B. innocens* B256^T
SHI-068	710	100.0	*B. hampsonii* NSH-16^T	97.8	*B. murdochii* 56-150^T	97.7	*B. innocens* B256^T
T049	792	94.0	*B. murdochii* 56-150^T	98.0	*B. murdochii* 56-150^T	97.8	*B. innocens* B256^T
T054	792	100.0	*B. innocens* B256^T	98.2	*B. murdochii* 56-150^T	98.0	*B. murdochii* 56-150^T
T057	792	95.7	*B. intermedia* PWS/A^T	99.6	*B. murdochii* 56-150^T		
T062	791	99.1	*B. murdochii* 56-150^T	99.5	*B. pilosicoli* P43/6^T		
T064	792	95.5	*B. murdochii* 56-150^T	100.0	*B. murdochii* 56-150^T		
T079	789	91.0	*B. murdochii* 56-150^T	97.8	*B. murdochii* 56-150^T	97.7	*B. innocens* B256^T
T103	792	98.4	*B. intermedia* PWS/A^T	99.3	*B. murdochii* 56-150^T		
TL_wh1	918	98.8	*B. hyodysenteriae* B78^T	100.0	*B. hyodysenteriae* B78^T		
TL_wh2	918	98.8	*B. hyodysenteriae* B78^T	99.3	*B. hyodysenteriae* B78^T		

[a] *Nox* sequences were analyzed using Megablast with default settings and the database Nucleotide collection nr/nt (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
[b] *Cpn60*UT sequences were analyzed using FASTA to query a database of *cpn60* nucleotide sequences (http://www.cpndb.ca/).
[c] size of the sequenced *nox* fragment; the *cpn60*UT always is 555 bp.
[d] According to Clinical and Laboratory Standards Institute guideline MM18-A (2008) *Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing* a sequence can be assigned to a certain bacterial species if it has ≥99% identity to the sequence of the type strain and > 0.8% separation from other species.

additionally harbored strongly haemolytic *B. hyodysenteriae* (isolate JR7 in combination with 3824-15x/19) and on the other farm weakly haemolytic *B. hyodysenteriae* were isolated in addition (isolate JR91 in combination with 2982-15x/17).

*Cpn60*UT sequencing proved to be a good alternative to *nox* sequencing for identification of *Brachyspira* species, with only few discrepancies. These discrepancies were resolved in support of the *cpn60* result by the use of specific PCRs for pathogenic species. *Cpn60*UT sequencing is a well standardized, labor and cost effective approximation for species assignment in the clinical setting where whole-genome sequencing and the resulting data analysis is still not readily handled. Considering the fluid nature of the species concept in prokaryotes (Alexander et al., 2015; Harayama and Kasai, 2006; Janda, 2018) and the extensive gene rearrangements within and between *Brachyspira* species (Hampson and Wang, 2018), it may have to be accepted that some isolates present with a mosaic genome and are not easily assigned to a species with any of the methods available.

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None

Declaration of Competing Interest

None

Appendix A. Supplementary data

Supplementary tables related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108454.

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