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Student Final Report

THE EPIDEMIOLOGY OF OVINE FOOTROT AND VIRULENCE AND PHYLOGENY OF *DICHELOBACTER NODOSUS* IN A SINGLE FLOCK AND GLOBALLY

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CONTENTS

1 Abstract

Footrot, caused by *Dichelobacter nodosus*, accounts for 70% of foot lesions in sheep in the UK and is a huge economic issue for the sheep industry. There are two clinical presentations: interdigital dermatitis and severe footrot where hoof horn separates from the underlying tissue. Neither *D. nodosus* load in relation to the severity within ID and SFR, nor the effects of footrot treatments on *D. nodosus* load, have been investigated. The phylogenetic diversity of *D. nodosus* and its key virulence factors also required deeper investigation as their association with clinical disease remain unclear.

D. nodosus load was investigated using molecular techniques, on the feet of 99 ewes from one farm in the UK. Each ewe was assigned one of two treatments; foot trimming plus topical antibiotics or parenteral plus topical antibiotics. Interdigital skin swabs of two feet of 25 of the ewes were analysed and *D. nodosus* load quantified using qPCR. Fimbrial serogroup and acidic protease *B2*/*V2* of *D. nodosus* were investigated through molecular and sequencing analysis. *D. nodosus* phylogeny was analysed at a single farm level and global level.

D. nodosus load increased with ID score but showed no relationship with SFR score. *D. nodosus* load decreased one week after treatment with either FTA or PTA, but not with parenteral antibiotics alone (administered for a separate health reason). This indicates that topical antibiotics reduced *D. nodosus* load on feet. *D. nodosus* detection, along with fimbrial serogroup and acidic protease *B2*/*V2* detection, was higher and more representative through molecular analysis than through culture. One of the three phylogenetic clades identified from the single farm data indicated that strain introduction occurred when new stock was brought onto the farm. From both the single farm data and global data, neither fimbrial serogroup nor acidic protease identification related to disease severity or pathogenicity.

2 Introduction

2.1 What is ovine footrot?

As of 2018 there are more than 22 million sheep in the UK (GOV.UK, 2018). Footrot is endemic in the UK with more than 90% of sheep farmers reporting footrot in their flocks (Winter *et al.*, 2015). Approximately 70% of lameness in sheep is from footrot and, as of 2015, approximately 5% of sheep are lame at any one time (Winter *et al.*, 2015). This is a positive reduction from 10% of sheep lame back in 2004 (Kaler & Green, 2008). Footrot leads to loss of body condition in affected sheep, reducing their productivity and ability to rear lambs successfully (Kaler & Green, 2008, Green *et al.*, 2007, Wassink *et al.*, 2010). Consequently, the economic impact of lameness to the UK sheep industry is estimated to range between £24 and £80 million per annum (Nieuwhof & Bishop, 2005, Wassink *et al.*, 2010). Hence lameness is one of the biggest concerns for farmers in the sheep industry. The first stage of footrot is interdigital dermatitis (ID) where the epidermis of the interdigital skin of the foot becomes inflamed (**Figure 1.1a**). This can progress to destruction of the epidermal matrix and separation of the hoof horn from the underlying sensitive tissue (**Figure 1.1b**) (Beveridge, 1941). This second stage is termed severe footrot (SFR) (Winter, 2008). The main causative agent of ovine footrot, *Dichelobacter nodosus*, is a Gram negative, motile, obligate anaerobe (Beveridge, 1941, Witcomb *et al.*, 2015).

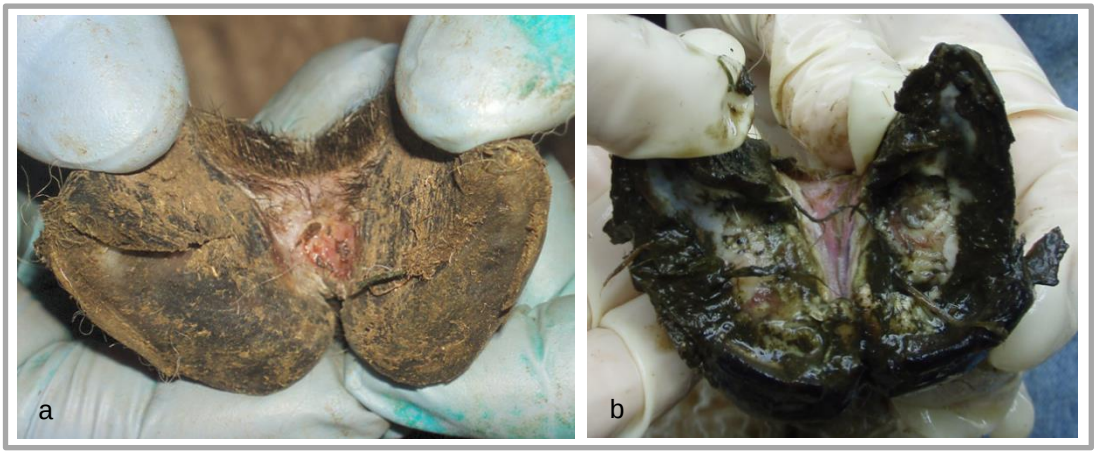


Figure 2.1: Examples of clinical interdigital dermatitis (a) and severe footrot (b).

Movement of sheep between UK farms is common and very few flocks are closed, making elimination of footrot extremely difficult in the UK (Green & George, 2008). From research over the past 10 years, recommended treatment for footrot is parenteral and topical antibiotics within three days of a sheep becoming lame (Wassink *et al.*, 2010, Kaler *et al.*, 2010b, Winter *et al.*, 2015). Separation of affected sheep is key whilst trimming the hoof horn is unnecessary if not detrimental to the recovery process (Wassink *et al.*, 2004, Wassink *et al.*, 2003, Kaler *et al.*, 2010b). Hoof horn

overgrowth occurs as a consequence of lameness with footrot. It does not cause the development of footrot as previously thought. Hoof horn recovers to a healthy state after treatment as recommended (Smith *et al.*, 2014, Kaler *et al.*, 2010b).

2.2 Pathogenesis of footrot

If the interdigital skin between the toes becomes damaged or continuously wet this facilitates entry of *D. nodosus* and other secondary pathogens like *Fusobacterium necrophorum* into the hoof (Beveridge, 1941, Graham & Egerton, 1968). *D. nodosus* is then able to degrade tissue with the use of key virulence factors described later in this chapter. An increase in *D. nodosus* load has been linked with an increase in the severity of the inflammatory response (Agbaje *et al.*, 2018). Higher loads (number of cells) of *D. nodosus* have been detected prior to and during ID, followed by a slight drop in load if severe footrot occurred but still higher than the load detected on healthy feet (Witcomb *et al.*, 2014, Calvo-Bado *et al.*, 2011b). By the severe footrot stage the epithelium is shed from the interdigital skin, hence the reduced detection of *D. nodosus* on the surface of the interdigital skin than that detected in ID (Witcomb *et al.*, 2015). Disease severity is also associated with an increased load of *F. necrophorum* prior to severe footrot, with the population of other bacteria in the environment remaining relatively stable regardless of disease state (Witcomb *et al.*, 2014). *F. necrophorum* is an opportunistic secondary invader associated with severe footrot but *D. nodosus* is considered as the driver of the disease (Beveridge, 1941, Witcomb *et al.*, 2014).

2.3 Effect of climate, the environment, sheep age and breed on footrot

Climate is a risk factor in the UK and other countries, with prevalence of footrot seen to increase with increasing rainfall and temperature (Smith *et al.*, 2014). *D. nodosus* had previously been found to survive in soil for up to 14 days and up to 40 days in microcosms (Cederlof *et al.*, 2013, Muzafar *et al.*, 2016). A more recent study has further highlighted the importance of moisture in the persistence of *D. nodosus*. Without moisture, *D. nodosus* is likely to only persist for a few days on pasture (Clifton *et al.*, 2019). *D. nodosus* survival at 5°C has also been recorded; a lower temperature than previously thought (Cederlof *et al.*, 2013).

Pasture with a longer sward length is more likely to enable a more humid climate close to the soil and so could lead to an increased risk of weakening the interdigital skin and lead to disease (Angell *et al.*, 2018). But equally problematic could be heavily grazed pasture where exposed soil may become poached which could also damage the interdigital skin (Angell *et al.*, 2018) allowing *D. nodosus* to penetrate. The feet of sheep are in constant contact with the pasture and so cross-contamination with *D. nodosus* between the two is highly likely (Clifton *et al.*, 2019). Feet however

are the most significant reservoir of *D. nodosus* (Clifton *et al.*, 2019). With diseased feet driving the persistence of *D. nodosus* transmission (Clifton *et al.*, 2019).

Susceptibility to lameness has also been associated with increased age (Kaler *et al.*, 2010b). Age however may be confounded by other management strategies such as separating yearling lambs (Angell *et al.*, 2018). This may have a biosecurity effect in reducing the spread of footrot which is more often seen in older sheep (Kaler *et al.*, 2010b). All breeds of sheep are susceptible to footrot. But again, breed may be confounded by management strategies, with hill breeds often in lower stocking densities than lowland breeds for example. This makes the spread of *D. nodosus* to other sheep is less likely.

2.4 Footrot lesion scoring

Scoring of footrot lesions varies across countries. The scoring system devised by Egerton & Roberts (1971) and used in Australia is given in **Table 1.1**. In Sweden a score of 0 or 1 is considered healthy, a score of 2 is a foot with moderate footrot, and a score of 3 to 5 is a foot with severe footrot (Frosth *et al.*, 2015). Switzerland is similar with some aspects of Egerton & Roberts (1971). In the UK the scoring system devised by Moore *et al.* (2005), and further detail added by Kaler *et al.*, 2010b, is more frequently used and is given in **Table 1.2**. This system allows for a separate score for interdigital dermatitis and severe footrot lesions as lesions may be observed at one stage and not the other. This can give more informative detail. The differences in how footrot lesions are scored across countries can make comparisons more problematic at the early stages of disease but overall records are comparable (Foddai *et al.*, 2012).

Table 2.1: Description of lesion scores devised by Egerton and Roberts (1971).

Score	Description
1	Mild interdigital dermatitis
2	More extensive interdigital dermatitis
3	Severe interdigital dermatitis and under-running of the horn of the heel and sole
4	Severe interdigital dermatitis and under-running of the horn of the heel, sole and wall

Table 2.2: Description of lesion scores devised by Kaler *et al.* (2010b).

Score	Description
Interdigital dermatitis	
0	Undamaged, no lesion
1	Slight interdigital dermatitis, irritation of the skin, but dry
2	Slight interdigital dermatitis with a fetid smell, < 5% skin affected
3	Moderate interdigital dermatitis with a fetid smell 5-25% skin affected
4	Severe interdigital dermatitis with a fetid smell > 25% skin affected
Severe footrot	
0	Undamaged, no lesion
1	Active or healing footrot lesion with a degree of separation of the sole of the digit
2	Active footrot lesion with a marked degree of separation of the sole of the digit
3	Active footrot lesion with extensive under-running of the hoof wall (may include under-running of the sole)
4	Active footrot lesion with complete under-running of the hoof wall (may include under-running of the sole)

2.5 *Dichelobacter nodosus* detection

Dichelobacter nodosus is difficult to culture and difficult to detect (Frosth *et al.*, 2012). Detection was made easier when a PCR targeting the 16S rRNA gene of *D. nodosus* was developed by (Lafontaine *et al.*, 1993) and then improved upon by Belloy *et al.* (2007) with a nested PCR. Pooling of foot swab samples is a common way of establishing flock level detection of *D. nodosus* (Frosth *et al.*, 2017, Greber *et al.*, 2018). However, sensitivity can be compromised if, for example, a positive sample with a low load of *D. nodosus* diluted by negative samples may not be detected (Frosth *et al.*, 2017). But pooling could benefit swifter detection at flock level. Currently the most effective method of detecting *D. nodosus* is through quantitative PCR. This approach targets the *rpoD* gene which is a single copy making this method more sensitive than targeting the 16S rRNA gene (Calvo-Bado *et al.*, 2011b).

The fact that *D. nodosus* is motile and has recently been found below the surface of the soil, suggests it is more than likely that *D. nodosus* is able to invade deeper tissue and cause chronic infection (Clifton *et al.*, 2019, Beveridge, 1941). Witcomb *et al.* (2015) extracted DNA from punch biopsies (post mortem) of the interdigital skin and both *D. nodosus* and *F. necrophorum* were found in the dermal layers of sheep feet. However the sample size was very small with four feet of only six sheep sampled (Witcomb *et al.*, 2015). The invasion of deeper tissue could provide a route of entry allowing other pathogens as well as *F. necrophorum* to enter and drive the infection further (Roberts & Egerton, 1969).

2.5.1 The conflict between investigating deeper tissue at one time point (culled sheep) or surface analysis over time

Many studies have investigated footrot at one time point, i.e. on the feet of animals either culled or from abattoirs. For instance, Belloy *et al.* (2007) compared detection of *D. nodosus* on Ibex and sheep using animals that were either shot or obtained from an abattoir. This allows for greater exploration into deeper tissue to determine where *D. nodosus* is detectable than would be ethically possible with live animals. It has also led to the question of by what means does *D. nodosus* penetrate tissue? However, problems with samples from these sources include sample contamination or decomposition which may change the microbial community potentially biasing detection results. The load of bacteria on the feet of culled sheep may be altered as a result of being transported to the laboratory or other facility prior to processing. Added to this, a foot obtained from an abattoir will have minimal if any background information with it on the sheep or farm it came from other than the state of the feet at the time of death or time of analysis. Thus, making the use of subsequent data reasonably restrictive. Another clear limitation with samples of this nature is the inability to monitor changes or recovery from footrot over time. Prevalence of footrot at a single time point is also unlikely to be an accurate representation of disease levels in a flock (Angell *et al.*, 2018). Longitudinal data would be more informative and facilitate better our understanding of disease progression. A clearer understanding of how the presence and load of *D. nodosus* changes over time and how this reflects the severity of footrot is needed.

2.6 The differentiation between strains of *Dichelobacter nodosus* considered virulent and benign

The distinction between virulent and benign strains of *D. nodosus* is still not definite. A problem with differentiating between virulent and benign *D. nodosus* strains is that often multiple strains are isolated on the same foot (Cheetham *et al.*, 2006, Cagatay & Hickford, 2011, Gradin *et al.*, 1993). So apparently benign strains may be detected on farms or feet with severe footrot (Cheetham *et al.*, 2006, Cagatay & Hickford, 2011). Benign strains have been detected on feet with clinical signs of severe footrot (Frosth *et al.*, 2015). Plus, virulent strains have been isolated on feet with no clinical signs of disease (Locher *et al.*, 2015). Virulent strains also appear varied in phenotype (McPherson *et al.*, 2017). As a result, the distinction between virulent and benign is unclear and needs investigating further. In addition, linking strains with a scale of virulence or level of disease provides a greater challenge that has not been investigated in depth (Moore *et al.*, 2005).

2.7 Key virulence factors

Bacteria can develop a number of enzymes or appendages to help colonise an environment. A number of factors have been identified in *D. nodosus* but their association with virulence has varied in both their role and the certainty of their contribution towards virulence as shown in the latter part of this literature review.

2.7.1 Type IV fimbriae and fimbrial biogenesis

Type IV fimbriae are mentioned often amongst *D. nodosus* virulence factors as these appendages are needed for protease secretion in protein breakdown (Han *et al.*, 2008, Myers *et al.*, 2007). The fimbrial subunit gene of type IV fimbriae, *fimA* is needed for fimbrial biogenesis referring to natural transformation and cell or surface adhesion (Kennan *et al.*, 2011, Kennan *et al.*, 2014). Fimbriae are highly immunogenic and *fimA* is the first factor used to classify serogroups of *D. nodosus* (Kennan *et al.*, 2011, Myers *et al.*, 2007). Serogroups group strains with a common antigen that is recognised by the same specific antibody. Serogroups can be further subdivided by serotype based on specific surface antigens. Ten serogroups and 18 – 20 subsequent serotypes have been identified so far in *D. nodosus* (Dhungyel *et al.*, 2002, Ghimire *et al.*, 1998, Kumar *et al.*, 2016). Bhat *et al.* (2013) identified a *D. nodosus* strain with fimbriae which differed to the fimbriae of known serogroups, showing that methods of serotyping still need refining. Also, recently McPherson *et al.* (2018) highlighted that culture-based methods are unlikely to pick up all serogroups present in a flock. It is therefore important to be aware when identifying serogroups that more groups previously undetected may be found in the future.

Serogroups A, B, C, E, F, G, I and M and serogroups D and H have been linked with virulent and benign strains respectively and are regarded as a key virulence factor (Kennan *et al.*, 2011, Kennan *et al.*, 2014, Claxton *et al.*, 1983). Although Kennan *et al.* (2014) observed that fimbriae are seen as essential for virulence, none of the fimbrial genes were present exclusively in virulent strains. Links between particular serogroups and virulence have varied with country and outbreak. Simply identifying the serogroups does not definitively distinguish virulent and benign strains (Dhungyel *et al.*, 2002). This again highlights the lack of a clear distinction between virulent and benign strains.

2.7.1.1 Regulation of movement

Chemosensory systems are utilised by bacteria to facilitate chemotaxis and so colonise new environments. A number of two-component regulatory systems have been identified in *D. nodosus* including *PilSR* (regulates the expression of *fimA*) and *twmSR* (Kennan *et al.*, 2015). The genes that encode these systems were identified in all 103 *D. nodosus* strains that were sequenced by

Kennan *et al.* (2014) (further described in **section 1.10**). The chemosensory system in *Pseudomonas aeruginosa* regulates twitching motility (surface movement), and so the similarity of the chemosensory system observed in *D. nodosus* with *P. aeruginosa* suggests that this is likely to regulate its twitching motility but is yet to be determined (Kennan *et al.*, 2015). Also, the signals that may initiate, or compounds that may influence, the direction of twitching motility in *D. nodosus* have not yet been identified (Kennan *et al.*, 2015). However, as twitching motility has been linked with virulence there is a strong possibility that these regulatory systems for twitching motility may influence the virulence of isolates and so have an impact on the pathogenesis of footrot (Kennan *et al.*, 2015, Depiazzi *et al.*, 1991).

2.7.2 Serogroups and their use in vaccine development

Vaccination is a strategy for footrot prevention and both monovalent- and polyvalent-serogroup vaccines have been developed depending on the number of serogroups the vaccine targets (Schwartzkoff *et al.*, 1993). The first commercial vaccines were developed in Australia by 1972 and were monovalent (Keogh, 1976). However, their efficacy was poor possibly due to multiple serogroups being present and so these vaccines were withdrawn in 1976 (Keogh, 1976, Dhungyel *et al.*, 2014). Shortly after, multivalent vaccines became available (Keogh, 1976). Serogroups are used to formulate vaccines against footrot, so an unclear distinction of virulence is problematic when trying to identify truly virulent and benign strains hence making the design of appropriate vaccines more difficult. If there are more than two serogroups involved, footrot can be controlled but multiple vaccinations may be needed (Dhungyel *et al.*, 2013a).

The necessity for multiple vaccinations relates to varied efficacy possibly due to how close antigens of detected strains relate to one another and also antigenic competition to the immune response can occur (Schwartzkoff *et al.*, 1993, Hunt *et al.*, 1995). In the UK, around 10% of farmers stated they vaccinated at least once a year in 2015 (Winter *et al.*, 2015). A study of a single UK flock found that use of targeted antibiotics plus two doses of the footrot vaccine (Footvax, MSD) yielded a higher recovery rate than antibiotics alone (92% compared with 82%) (Duncan *et al.*, 2012). This corroborates with a 20% reduction in lameness reported with the use of the footrot vaccine alongside other management strategies mentioned (Winter *et al.*, 2015). An overall vaccine efficacy of 62% against footrot was identified by Duncan *et al.* (2012).

Antigenic competition is a factor to be considered when designing appropriate vaccines (Hunt *et al.*, 1995, Schwartzkoff *et al.*, 1993). It was previously thought that a vaccine with a broader range of serogroup specific content would provide more effective protection (Thorley, 1986). However since antigenic competition has been observed, such variation in the fimbrial gene can effect antibodies

binding and so affect the efficacy of a vaccine (Cagatay & Hickford, 2005). Little is known about the most recently identified serogroup, M or about its pathogenicity (Ghimire *et al.*, 1998, Dhungyel *et al.*, 2015). If a vaccine containing serogroups A-I is used, a sheep's immune response may not be optimal against any or all of the serogroups detected in an outbreak (Hunt *et al.*, 1995, Schwartzkoff *et al.*, 1993, Dhungyel *et al.*, 2013a). Identifying the serogroups in a recent outbreak is key to developing the most effective vaccines for flocks in that area (Dhungyel *et al.*, 2002, John *et al.*, 1999).

2.7.3 Extracellular serine proteases (ESPs)

ESPs are another key virulence factor of *D. nodosus* having been linked with the breakdown of host proteins for a potential source of energy for protein synthesis (Stäuble *et al.*, 2014, Myers *et al.*, 2007). Higher protease activity has been associated with virulent *D. nodosus* strains (Depiazzi *et al.*, 1991). ESPs *aprV2*, *aprV5* (acidic subtilisin proteases) and *bprV* (basic subtilisin protease) have been identified as being from virulent strains of *D. nodosus* (Riffkin *et al.*, 1995). ESPs *aprB2*, *aprB5* and *bprB* have been identified as being from benign strains of *D. nodosus* (Riffkin *et al.*, 1995). Recent studies suggest that *D. nodosus* can be divided into two distinct clades which correlate with the 2bp substitution between the most noted virulence factors, *aprV2* and *aprB2* proteases of tyrosine (TAT) to arginine (CGT) respectively at position 661/662 (Kennan *et al.*, 2014, Riffkin *et al.*, 1995). Acidic protease *V2* is directly involved in the breakdown of keratin in the hoof horn (Kennan *et al.*, 2010) but both *B2* and *V2* elicit a host inflammatory response (Maboni *et al.*, 2017). The 2bp change between *aprV2* and *aprB2* has been found to correlate with individuals with clinically virulent severe footrot but this needs further investigation with varying footrot severity (Stäuble *et al.*, 2014). It is observed that virulent strains produce thermostable proteases, whereas benign strains produce considerably less thermostable proteases (Cheetham *et al.*, 2006, Depiazzi *et al.*, 1991, Palmer, 1993). This may be beneficial to virulent strains in their ability to establish and sustain infection. Recently a different amino acid at position 92 of *aprV2*/*B2* was found after running an *aprV2*/*B2* and 16S rRNA PCR assay to detect *D. nodosus* (Frosth *et al.*, 2017). This variant could also play a part in the virulence of *D. nodosus*.

Examples of protease tests include the gelatin gel test for protease thermostability and the elastase test for protease activity (Palmer, 1993, Stewart, 1979, Dhungyel *et al.*, 2013b). These tests require culture potentially making conditions harder to control and quantification more difficult as well as being dependent on sufficient growth being achieved (Palmer, 1993, Stewart, 1979, Dhungyel *et al.*, 2013b). These tests are also used to differentiate between virulent and benign strains. However, these tests are not sensitive enough to distinguish intermediate levels of virulence compared with the potential of PCR-based methods (Rood *et al.*, 1996). A study by Gilhuus *et al.* in 2013

characterised *D. nodosus* isolates by virulence but did not evaluate the correlation between detecting virulent *D. nodosus* strains (based on the presence of *aprV2*) and observations of lesions on sheep. Plus, this study found under-running of the horn in flocks with benign strains detected. Therefore, the lack of clear concurring results between laboratory tests and clinical signs makes their assumption of the importance of gelatin-gel testing for virulence weak.

2.8 Other virulence factors

There are virulence-associated proteins (*vap*) and virulence-related loci (*vrl*) associated with *D. nodosus* strains and linked with thermostability (Han *et al.*, 2008, Cheetham *et al.*, 2006). However, as yet the function of *vap* or *vrl* genes remains unknown (Myers *et al.*, 2007). Also, approximately 30% of *vap* islands are also found in benign strains (Han *et al.*, 2008, Cheetham *et al.*, 2006). The *integraseA* gene involved in DNA binding has a strong association with virulence and appears to be absent from benign *D. nodosus* strains (Cheetham *et al.*, 2006). A further problem with understanding the difference in virulent and benign *D. nodosus* strains is that often multiple strains are isolated, so apparently benign strains (no *intA* gene detected) may be detected on farms with virulent footrot (Cheetham *et al.*, 2006).

Progesterone receptor *(Pgr)*, a large repetitive protein secretion coding gene, is another virulence-associated region and has only been detected in virulent strains (Myers *et al.*, 2007). It has been suggested that *Pgr* encodes a virulence factor and could be involved in adhesion to the extracellular matrix (Gilhuus *et al.*, 2014, Myers *et al.*, 2007). It has been suggested that *Pgr* genes may be responsible for the *D. nodosus* variants that develop when multiple strains colonise an area (sheep foot) (Calvo-Bado *et al.*, 2011a). Three DNA fragments of the *D. nodosus* genome have been isolated and linked to virulence, pJIR313, pJIR314B and pJIR318 (Rood *et al.*, 1996). However, these along with the other virulence factors in this section are no longer thought to link with virulence.

2.9 *Dichelobacter nodosus* strain diversity

D. nodosus has a relatively small genome, 1.39Mb, however approximately 20% is derived from lateral gene transfer (LGT) (Myers *et al.*, 2007, Kennan *et al.*, 2014). Once infection takes hold *D. nodosus* strains can undergo natural transformation and homologous recombination (Calvo-Bado *et al.*, 2011a). This is a key strategy in adapting to an ecological niche and could be a reason for the incomplete knowledge around *D. nodosus* strains and how they relate to disease severity.

Multi-locus variable number tandem repeat analysis (MLVA) has been used to gain a more detailed understanding of the strain community from *D. nodosus* isolates. Russell *et al.* (2014) analysed 77

isolates from five countries and demonstrated geographical clustering of isolates from Great Britain, Australia and India. Profiles were determined for each isolate based on the number of repeats at each of the four chosen loci: DNTR02, DNTR09, DNTR10 and DNTR19 (Russell *et al.*, 2014). Genetic recombination within populations was indicated, highlighting that *D. nodosus* is capable of high strain diversity thus making the identification of virulent, intermediate or benign strains more challenging. This makes vaccine development all the more challenging but footrot can be controlled if strains are detected early when only a few sheep have become infected.

Buller *et al.* (2010) obtained 796 *D. nodosus* isolates from 303 farms across four states in Australia from 1976 to 2002 and analysed them using pulsed-field gel electrophoresis. The molecular types from farms in Western Australia were tested and half the farms showed genetic diversity of *D. nodosus* strains isolated, similar to results found in (Russell *et al.*, 2014). In addition, up to three different molecular types could be found on an individual foot on a number of farms in Western Australia (Buller *et al.*, 2010). The identification of genetically similar molecular types could reflect the environmental conditions at the time potentially leading to competition between strains (Buller *et al.*, 2010). This compared to genetically diverse molecular types which could be as a result of transmission between sheep potentially bought in (Buller *et al.*, 2010). Similarly, Cagatay & Hickford (2011) detected different *D. nodosus* strains on different feet of the same sheep from the same farm showing strain diversity at foot level.

Three common clonal groups were found to dominate in infected sheep across all four states in Australia (Buller *et al.*, 2010). The finding of common clonal groups in Australia could be explained by the British colonisation towards the end of the 18th Century, a likely source of footrot being introduced. Also, the importation of the Spanish Merinos, a popular breed in Australia, as well as imports from other European countries may be the reason for the high genetic diversity (Buller *et al.*, 2010). Importation of sheep is less common in Great Britain but there is still a biosecurity risk from sheep being bought in nationally from around the UK. Good hygiene practises like quarantining and running a thorough health check on new stock is therefore a necessity for all farms (Green *et al.*, 2007).

The most prominent new outbreak of footrot was in Norway in 2008 with the cause indicated to be from a virulent strain of serogroup A being introduced (Gilhuus *et al.*, 2014, Meling & Ulvund, 2009). This introduction could have come from recent imports largely from Denmark in 2006 and 2007 or potentially from Germany or Finland in 2006 (Statistics Norway, 2019). The dominant virulent serogroup did not always remain the same in four of the seven flocks sampled more than once with footbathing occurring in between sampling points. This suggested changes in the dynamics of the

D. nodosus community possibly as a result of decontamination processes or from new sheep brought on to the farm (Gilhuus *et al.*, 2013).

Also from the Gilhuus *et al.* (2013) study, serogroups A, B, C, E, G, H and I were identified from benign isolates, contrary to serogroups D and H being associated with benign isolates (Kennan *et al.*, 2011). No more than three serogroups were detected in a single flock and only one serogroup was detected on the majority of individuals by multiplex PCR (Gilhuus *et al.*, 2013). This indicated that single-strain infections were common. The greater diversity in serogroups identified and geographical location in benign isolates suggested that the benign strains had been present for some time in Norway. Contrary to Gilhuus *et al.* (2013) up to seven serogroups may be identified in one flock (Dhungyel *et al.*, 2013a). Dominant *D. nodosus* strains have been detected repeatedly over time in a flock (Smith *et al.*, 2017). Also, infection of the foot with multiple *D. nodosus* strains is common (Calvo-Bado *et al.*, 2011b). However, more severe footrot has been seen in flocks with single-strain infection than in multi-strain infections (Gilhuus *et al.*, 2013, Ghimire *et al.*, 1996, Dhungyel *et al.*, 2013a).

2.10 The use of sequencing techniques to further the understanding of *Dichelobacter nodosus* in the pathogenesis of footrot

The recent advances of sequencing techniques like multi-locus sequence typing and whole genome sequencing have enabled further investigation into the *D. nodosus* genome. Most notable firstly is the study by Kennan *et al.* (2014) who compared the virulence of 103 globally sourced *D. nodosus* whole genome sequences. They found the genomes to be highly conserved and also clustered into two distinct clades (**Figure 1.2**). These clades were distinguished by acidic protease *B2* and *V2* identification, although how *aprB2*/*V2* were identified was not stated. The patterns of both sequence identity and differences were attributed to horizontal/lateral gene transfer. They also found isolates from the same outbreak were more closely related. Both clades appeared to be able to coexist on the hoof.

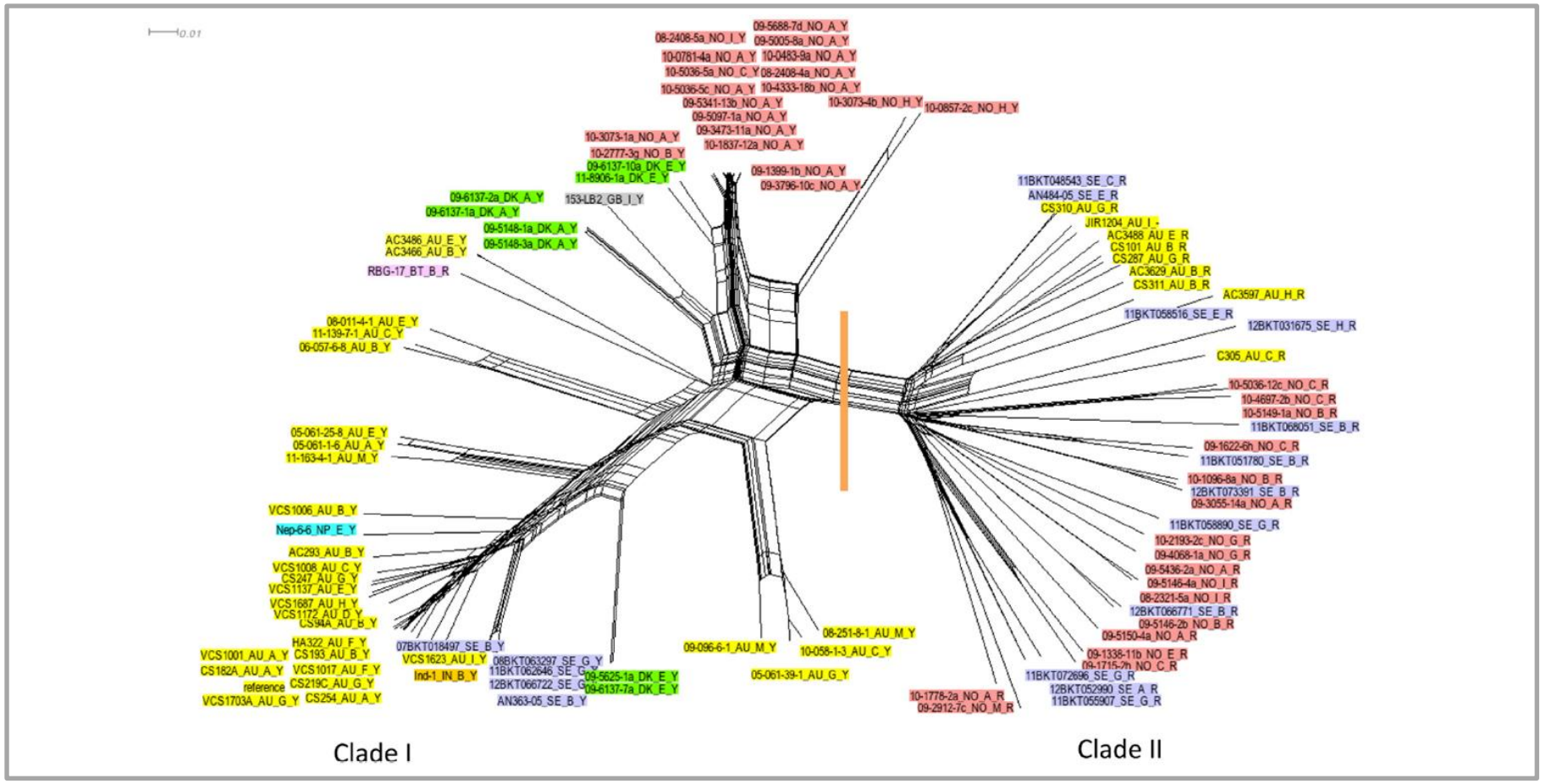


Figure 2.2: Network diagram from Kennan *et al.* (2014) showing 103 *D. nodosus* isolates comprising 31,627 bases from each isolate and the relationship inferred using the parsimony-based method. Clade I associated with detection of *aprV2* and clade II with *aprB2*.

Isolates coloured by country, Australia; yellow, Norway; red, Sweden; lilac, Denmark; green, Bhutan; pink, Nepal; blue, India; orange, UK; grey.

Secondly is the study by Blanchard *et al.* (2018) which devised a tiered multi-locus sequence typing (MLST) scheme (Blanchard *et al.*, 2018). Their analysis of isolates from the Kennan *et al.* (2014) study, and a further 67 *D. nodosus* isolates from the UK, yielded 115 strain types correlating mostly with country of origin. The ratio of strain type to number of isolates suggested a diverse collection of isolates but a low level of recombination. This is in contrast to previous studies where a high level of diversity and recombination have been observed (Kennan *et al.*, 2014, Russell *et al.*, 2014). The Blanchard *et al.* (2018) study did not mention *aprB2*/*V2* in their results. Further investigation into the phylogenetic relationships of *D. nodosus* and how it may link with clinical disease is needed.

2.11 Conclusions

The high genetic diversity often found in *D. nodosus* isolates provides a challenge for identifying virulent strains. But the finding that often a select number of strain types can dominate highlights the importance of strain detection early on in an outbreak, strategic use of vaccines, and separating diseased animals. The strain community and its associations with the scale of disease severity need investigating to gain a better understanding of the pathogenesis of footrot and how best to manage an outbreak. The finding that some genes noted as virulence factors are not exclusive to virulent strains adds to the complexity and uncertainty of knowledge surrounding *D. nodosus* strains. Also, the knowledge around initiation and regulation of twitching motility in *D. nodosus* is sparse. There is also little known about the regulatory systems used by *D. nodosus* to move and colonise new environments. The phylogenetic diversity and virulence factors of *D. nodosus* are proving varied and complex and it is important to investigate them further in order to improve our understanding of how *D. nodosus* establishes infection in sheep feet.

2.12 Aims for the current study

- To gain a clearer understanding of how the load of *D. nodosus* changes over the duration of disease and how this reflects the severity of lesions observed.
- To use non-culture-based techniques to detect the presence of key virulence factors of *D. nodosus*.
- To use whole genome sequencing to gain a clearer understanding of the key virulence factors of *D. nodosus*, namely *aprV2* and *aprB2* and *fimA* defining the serogroups, and how they associate with the severity of disease.
- To compare laboratory and genomic detection of these key virulence factors to understand better how genetic elements link with clinical disease.
- To investigate the phylogenetic diversity of *D. nodosus* strains and associations with disease severity.

3 Materials and methods

3.1 **Detection of *Dichelobacter nodosus* and associations between load and the severity of footrot**: Bristol Study flock samples

3.1.1 The study flock of 99 ewes, original study design and data collected

The samples for the current study were collected and stored during a previous farm study conducted from October 2010 to August 2011. That study has to date led to publications on hoof horn growth and *D. nodosus* strains from cultured isolates (Smith *et al.*, 2014, Smith *et al.*, 2017). The following farm and flock information was sourced from King, 2013. The farm was a lowland farm at the Department of Clinical Veterinary Science at the University of Bristol. The farm had a history of lameness caused by footrot. The commercially run flock was a closed flock until 1998 when new stock were brought in for research purposes. Since then the only movements in or out of the flock were purchased rams. The last introductions prior to the study period were three rams brought in from three different farms in 2008; a Texel ram from Bangor North Wales, a Hampshire ram from Somerset and a Suffolk ram from Somerset (three different farms). The flock was used for research and teaching purposes, although no other research was conducted on this flock during the study when the swabs were collected. The flock did not receive the footrot vaccine prior to or during the current study.

The flock, comprising 99 Mule and Suffolk-cross ewes and their spring lambs, was studied from mid-October 2010 to the end of August 2011. The three rams above were mated with the ewes in the autumn of 2010 shortly after the start of the Smith *et al.* (2014) study.

From here onwards this flock is referred to as the 'Bristol study flock'.

Ewes in the Bristol study flock were divided into two groups by stratified random sampling based on body condition from 0 to 5 (Defra, 1997), age, ID and SFR lesion score, and foot conformation score (Foddai *et al.*, 2012, Moore *et al.*, 2005). Each group was assigned one of two treatments for ewes if they developed footrot; either foot trim plus topical antibiotic foot spray (FTA) or parenteral antibiotics plus topical antibiotic foot spray (PTA). Ewes were locomotion scored each week and if a ewe in the FTA group had a locomotion score > 2 (minimum lameness was holding a foot up when standing, but weight bearing when walking as defined by Kaler *et al.*, 2009), all four feet were trimmed to remove any overgrowth and under-running horn and she also received topical application of oxytetracycline to all four feet. If a ewe in the PTA group became lame with a locomotion score > 2, she was examined, foot lesion scores recorded, and then she received 2000mg oxytetracycline (10mL Oxytetrin 20 LA [200mg/mL]; Intervet UK) by intramuscular injection and topical application

of oxytetracycline (Engemycin Spray; Intervet UK) to all four feet. Ewes also received their respective treatment if they had a SFR score > 2 (Beveridge *et al.*, 1941, Moore *et al.*, 2005, Foddai *et al.*, 2012) in accordance with the study's ethical statement (Smith *et al.*, 2014). The locomotion and foot lesions of all four feet of ewes that received treatment were scored once a week for the subsequent two weeks after treatment, with the exception of the first eight weeks of pregnancy when ewes were not caught (November and December 2010).

Four 4-week routine sampling periods were selected; October/November 2010, January 2011, May 2011 and August 2011. All 99 sheep were examined and sampled at the start of the first week of routine sampling period 1 (Oct 2010) and the start of the first week of routine sampling period 4 (Aug 2011). From the study flock of 99 ewes, 24 from the FTA group and 26 from the PTA group were selected for more detailed study. These 50 ewes were locomotion scored, and all four feet lD and SFR lesion scored, integrity scored and swab samples of the interdigital skin and any lesions taken each week of each four 4-week routine sampling period. The classification of locomotion scores, ID scores and SFR scores are described in **Table 2.1**.

Foot region refers to where a swab was taken on the foot; either from the interdigital skin or from a non-interdigital lesion at another part of the hoof.

Table 3.1: Definition of locomotion scores (Kaler *et al.*, 2009), ID scores and SFR scores (Beveridge, 1941, Moore *et al.*, 2005).

Score	Description
Locomotion scores	
0	Clinically sound, even weight distribution on all four feet
1	Mildly lame, slightly uneven gait and slight shortening of stride
2	Moderately lame, noticeable nodding of head, uneven gait, shortened stride
3	Badly lame, excessive nodding, holds up affected limb(s) while standing and obvious discomfort but putting foot to ground when moving
4	Severely lame, excessive nodding, holding up affected limb when standing and moving
5	As score 4 plus extreme difficulty rising, reluctant to move once standing and multiple limbs affected
6	Will not stand or move
Interdigital dermatitis lesion scores	
0	Clean interdigital skin with no dermatitis lesions or fetid smell
1	Slight interdigital dermatitis, irritation of the skin, but dry
2	Slight interdigital dermatitis with a fetid smell, < 5% skin affected
3	Moderate interdigital dermatitis with a fetid smell, 5-25% skin affected
4	Severe interdigital dermatitis with a fetid smell, > 25% skin affected
Severe footrot lesion scores	
0	Clean digit with no lesions
1	Active or healing lesion with a degree of separation of the sole of the digit
2	Active severe footrot lesion with a marked degree of separation of the sole of the digit
3	Active severe footrot lesion with extensive under-running of the wall hoof horn in the digit (may include under-running of the sole)
4	Active severe footrot lesion with complete under-running of the wall hoof horn in the digit (may include under-running of the sole)

Interdigital dermatitis: The first stage where the epidermis in the interdigital skin of the foot becomes inflamed.
Severe footrot: The second stage leading to destruction of the epidermal matrix and separation of the hoof horn from the underlying sensitive tissue.

In routine sampling periods (during one of the four 4-week periods as explained) or targeted sampling periods (outside the four 4-week routine sampling periods) if a ewe was recorded with a locomotion score > 2 it received its designated treatment. In addition, if any ewe from the Bristol study flock of 99 was observed to have a locomotion score > 2 at any point in the study (either in the routine or targeted sampling periods), it was lesion scored and received its designated treatment. A timeline of data collection that took place through the duration of the study is shown in **Figure 2.1**.

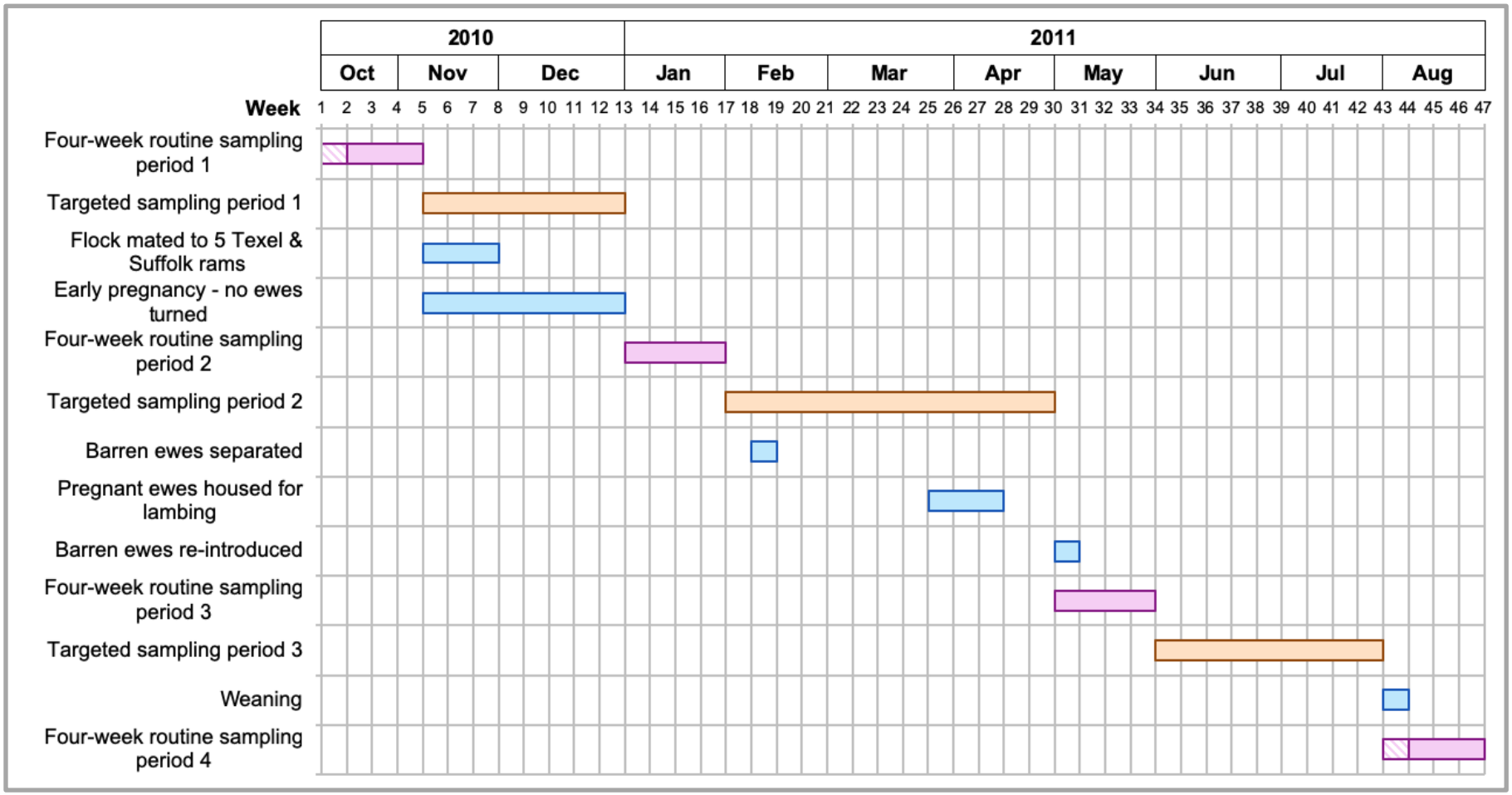


Figure 3.1: Key events over the study period.

Pink: Four-week routine sampling periods where the 50 ewes selected were sampled; *pink stripe*: study flock of 99 ewes were sampled; *orange*: targeted sampling periods; *blue*: husbandry events.

Two swab samples were taken simultaneously from each foot; a sterile wooden stick was used for one and a sterile cotton swab used for the other. Wooden stick samples were used to culture *D. nodosus* as described in (Smith *et al.*, 2017). Up to six individual *D. nodosus* colonies were selected from each positive culture and stored at 4°C. Cotton swab samples were stored in cryotubes containing 200µl phosphate-buffered saline (PBS) at -80°C for future DNA analysis.

3.1.2 Data selection for the current study

For the current study, 25 of the 50 detailed Bristol study ewes were selected; 12 from the FTA group and 13 from the PTA group. Two feet of the subset of 25 ewes were selected based on prioritising feet with isolates (Smith *et al.*, 2017) followed by choosing the two most and least diseased feet based on high and low occurrence of disease over time. An example of ID and SFR score and treatments given over the study period for all four feet for one of the 25 ewes is given in **Figure 2.2**. The flow chart to selection of swabs for the current study is presented in **Figure 2.3**. A breakdown of the origin of samples for each chosen ewe and when they were extracted is given in **Appendix 1**.

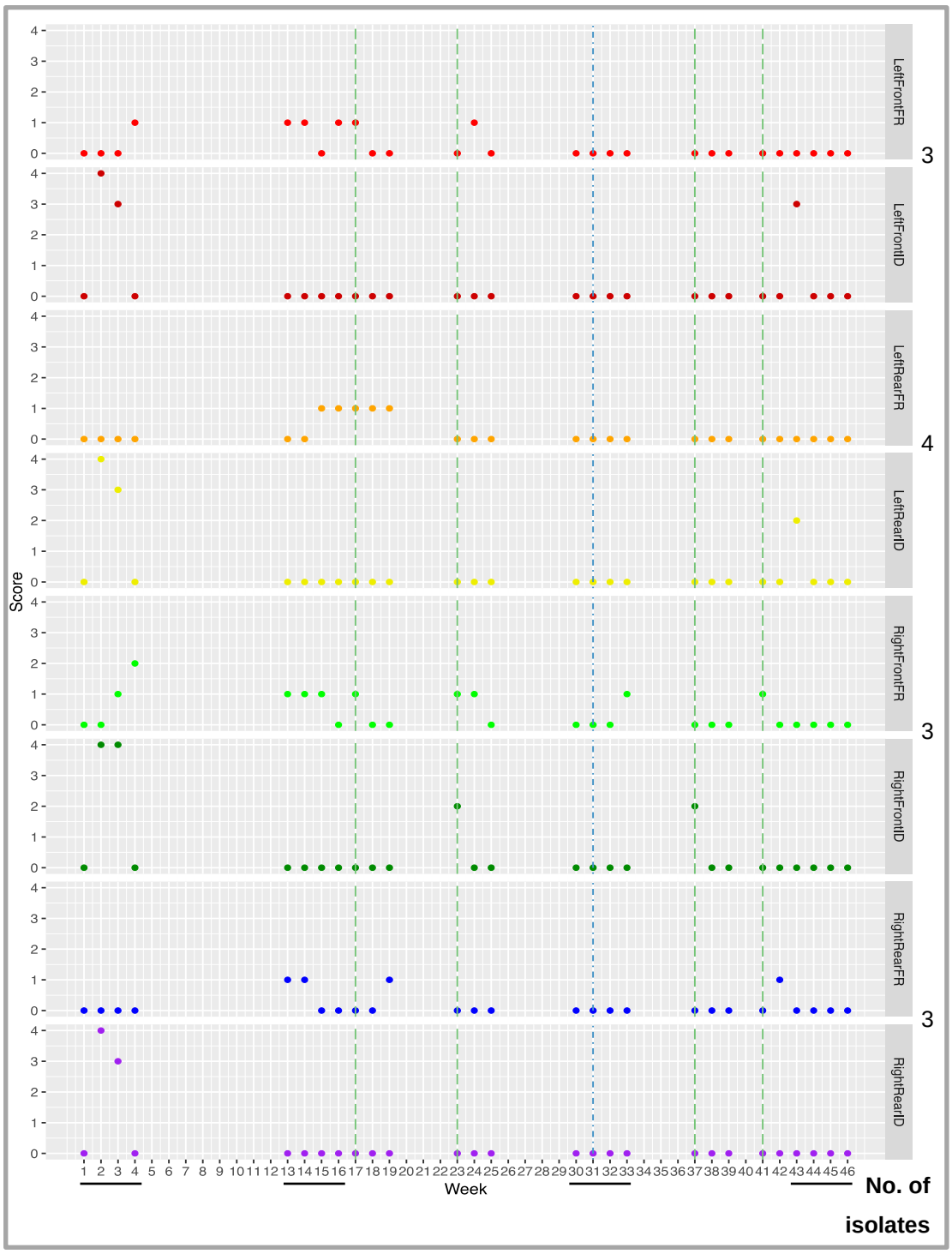


Figure 3.2: Disease profile for one of the 25 ewes selected that was in the PTA treatment group.

A separate score for ID and SFR from 0 to 4 is shown for each foot labelled on the righthand side with the number of isolates obtained for each foot. Weeks: routine sampling weeks, *green dashed line*: PTA treatment was given, *blue dot dashed line*: a different treatment was given for another health reason. For this ewe, the right front and right rear feet were selected as the 'most diseased' and 'least diseased' foot respectively for having the highest and lowest occurrence of disease.

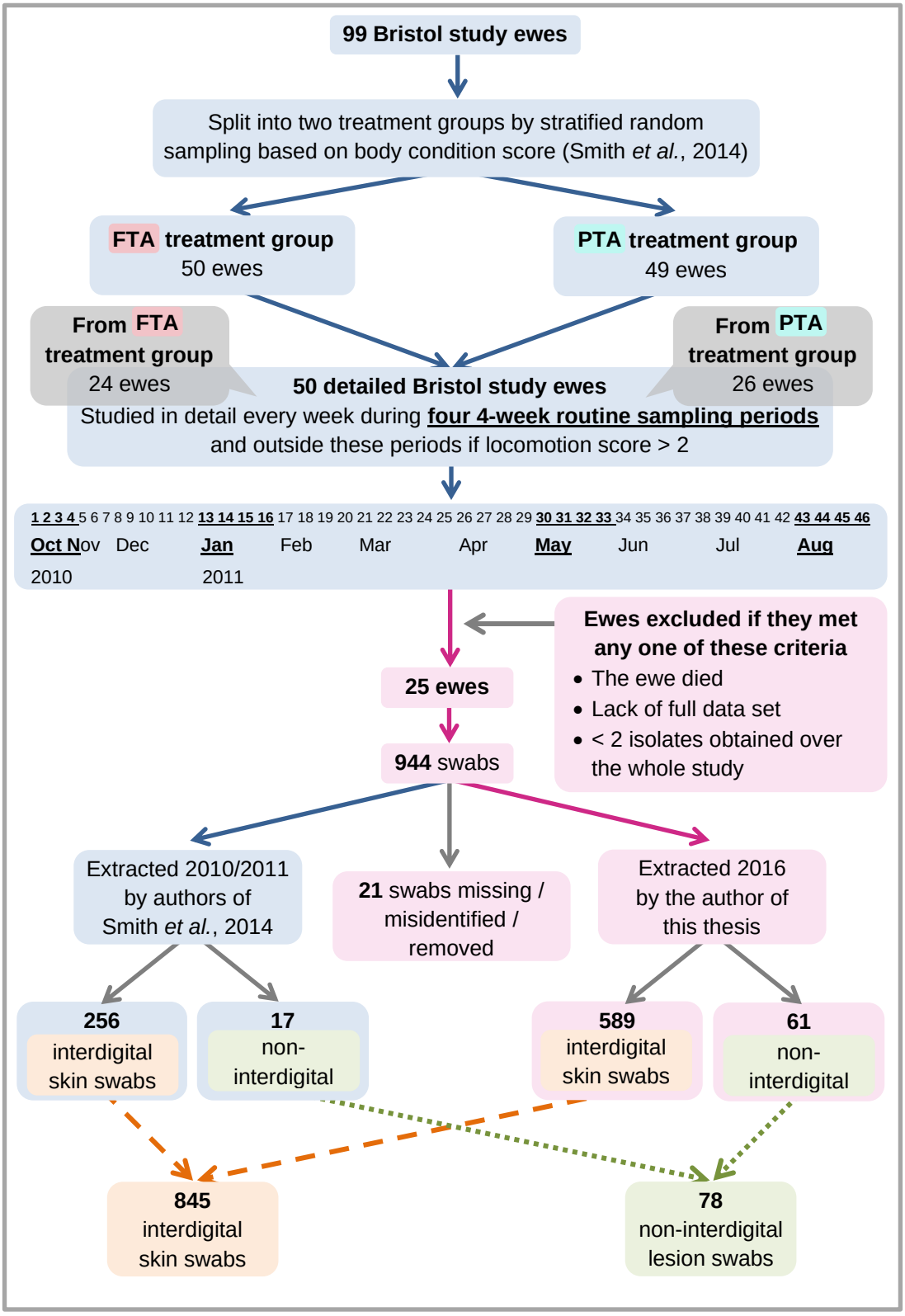


Figure 3.3: Origin of the subset of 25 ewes and swabs selected for analysis.

Blue: work done as part of previous studies Smith *et al.* (2014) and Smith *et al.* (2017), *pink*: work done by the author of this thesis.

3.1.3 Foot swab DNA extraction

Swab DNA was extracted from 650 interdigital skin and non-interdigital lesion swabs, with a negative control (200µl water) for each extraction batch, using the *Macherey-Nagel* NucleoSpin® tissue column kit detailed below. The DNA from the 273 swab samples extracted in 2010 and 2011 was also extracted following this method.

Four hundred microlitres T1 lysis buffer and 40µl proteinase K were added to each swab sample (stored in 200µl phosphate-buffered saline) plus one negative control (300µl water), vortexed, and incubated at 56°C for 10 minutes. Then 400µl B3 lysis buffer were added and the samples vortexed and incubated at 70°C for five minutes. The samples were allowed to cool for a further five minutes then centrifuged at 12,000 *g* for one minute to pellet debris. The supernatant was transferred to a 1.5ml microcentrifuge tube containing 400µl absolute ethanol. The tubes were centrifuged at 11,000 *g* for one minute. Seven hundred microlitres of each sample were loaded into a tissue column and the columns were centrifuged at 11,000 *g* for one minute. The flow-through was discarded and the remainder of the sample liquid was loaded into the tissue column and centrifuged at 11,000 *g* for one minute. The flow-through was discarded and 500µl BW wash buffer was added to each column and the columns were centrifuged at 11,000 *g* for one minute. The flow-through was discarded and 600µl B5 wash buffer was added to each column and the columns were centrifuged at 11,000 *g* for one minute. The flow-through was discarded and the columns were centrifuged once more at 11,000 *g* for one minute to dry the membrane. The tissue filter part of each column was placed into a 1.5ml microcentrifuge tube and 50µl pre-heated BE elution buffer was added to the membrane and left for two minutes. The filter columns together with the microcentrifuge tubes were then centrifuged at 11,000 *g* for one minute to elute the DNA. The tissue filter parts were discarded. Once eluted, 25µl of each sample was stored at -80°C and the remainder of each sample (~20µl) was stored at -20°C.

3.1.4 Quantitative PCR of extracted foot swab DNA

The 650 swab samples extracted together with 273 swab samples extracted in 2010 and 2011 were analysed by quantitative PCR (qPCR) following the method, thermal cycle profile and gene copy analysis stated elsewhere (Calvo-Bado *et al.*, 2011b). The reaction master mix composition was slightly different comprising of 1x Taqman® Universal PCR master mix (Promega), 0.9µM *rpoDF* primer, 0.9µM *rpoDR* primer, 0.25µM Taqman probe, 1mg/ml bovine serum albumin solution, 1µl DNA plus nuclease-free water resulting in a total reaction volume of 25µl. Primer sequences are given in **Table 2.2** and were synthesised by TIB MOLBIOL, GmbH, Berlin, Germany. A standard curve of *D. nodosus* strain VSC1703A was used to measure the number of *rpoD* gene copies of samples tested in triplicate, of which an average was taken (Calvo-Bado *et al.*, 2011b).

Table 3.2: Quantitative PCR primer and probe sequences (Witcomb *et al.*, 2014).

Primer/probe	Sequence 5' – 3'
rpoD forward primer	AACCTCCGGCAGAAGAAAAATT
rpoD reverse primer	CGTGAGGCATACGTAGAGAACTGT
TaqMan® probe	6FAM1-TCGAACATCTCTCGCTTTTTCCCCGA-BBQ

6FAM: 6 carboxy-fluoroscein.
BBQ: Black Berry Quencher.

The calculated *D. nodosus* standard dilutions used in the qPCR measurements are given in **Table 2.3**. Standard dilutions 3 – 9 and a negative control of water were used. Each standard/sample/negative control was tested in triplicate. The qPCR running programme consisted of a holding stage of 50° for two minutes then 95° for 10 minutes, followed by a cycling stage of 95° for 15 seconds then 55° for one minute for 40 cycles. Samples processed through qPCR were run and analysed using Applied Biosystems 7500 Fast Real Time PCR Systems software v2.0.6. The qPCR is able to detect down to one copy per microlitre of extracted DNA. Load per swab was calculated from load per microlitre and then logged to the base 10 then +1 to remove any resulting negative values.

Table 3.3: Quantitative PCR *Dichelobacter nodosus* standard dilutions.

Dilution	Source of plasmid DNA	Initial conc. (ng/µl)	Volume of plasmid DNA (µl)	Volume of dilutent (µl)	Final volume (µl)	Final conc. (ng/µl)	Resulting copy number (*rpoD*/µl)
1	Stock	8.8×10^{-8}	10	990	1000	8.8×10^{-10}	N/A
2	Dilution 1	8.8×10^{-10}	50	50	100	4.4×10^{-10}	1×10^{7}
3	Dilution 2	4.4×10^{-10}	10	90	100	4.4×10^{-11}	1×10^{6}
4	Dilution 3	4.4×10^{-11}	10	90	100	4.4×10^{-12}	1×10^{5}
5	Dilution 4	4.4×10^{-12}	10	90	100	4.4×10^{-13}	1×10^{4}
6	Dilution 5	4.4×10^{-13}	10	90	100	4.4×10^{-14}	1×10^{3}
7	Dilution 6	4.4×10^{-14}	10	90	100	4.4×10^{-15}	1×10^{2}
8	Dilution 7	4.4×10^{-15}	10	90	100	4.4×10^{-16}	1×10^{1}
9	Dilution 8	4.4×10^{-16}	10	90	100	4.4×10^{-17}	1×10^{0}

3.1.5 *Dichelobacter nodosus* isolate DNA from Smith *et al.* (2017)

The culturing process to obtain *D. nodosus* isolates from swabs from the Bristol study flock was described in Smith *et al.* (2017). DNA from these *D. nodosus* isolates was extracted previously to the current study using the *Macherey-Nagel* NucleoSpin® tissue column kit following the manufacturer's instructions (Smith *et al.*, 2017).

3.1.6 Statistical analysis

As this dataset had repeated measures from feet over time, statistical analyses allowing for dependence were used when appropriate. In all tests p values ≤ 0.05 were considered significant. Plots were created using MS Excel v16.29 or *RStudio* v0.99.903, *R* v3.3.1.

3.1.6.1 Anderson-Darling Normality Test (*Minitab Express* v1.5.0)

To calculate the distribution of load of *D. nodosus* positive samples.

3.1.6.2 Kruskal-Wallis Test (*Minitab Express* v1.5.0)

To test whether the median number of ewes with their respective number of cases of lameness were equal for the subset of 25 ewes and remaining 66 Bristol study ewes. To test whether the median number of ewes with their respective number of cases of lameness were equal for the two designated treatment groups; FTA and PTA.

3.1.6.3 Binomial mixed effects regression model (BM) for recovery from footrot by treatment type (*RStudio* v0.99.903, *R* v3.3.1)

Binomial mixed effects regression models were used to investigate the effect of treatment type on the reduction of positive locomotion scores and positive footrot lesion scores two weeks after treatment, i.e. reduction indicated recovery, separately accounting for multiple cases occurring in sheep. Footrot lesions were classed as 'reduced' if both positive ID and SFR scores (if both present) for a case of lameness had reduced two weeks after treatment. Otherwise the case of lameness was classed as 'not reduced'. Binomial mixed effects regression models were constructed using the 'glmer' function from the lme4 package in R (Bates *et al.*, 2015). The Akaike Information Criterion (AIC) was used to compare model fit and fixed effects were retained in the multivariable model if it resulted in a lower AIC.

3.1.6.4 Binomial mixed effects regression model (BM) for detection of *Dichelobacter nodosus* (*RStudio* v0.99.903, *R* v3.3.1)

Binomial mixed effects regression models were used to investigate the effect of locomotion score, ID score, SFR score, foot region, whether a sample was taken in a routine or targeted sampling period, if a sample was culture positive, and who had extracted the DNA on *D. nodosus* positive detection. Fixed effects were initially checked individually prior to running a multivariable binomial mixed effects regression model accounting for sheep, foot, foot region, week and who had extracted the DNA. Binomial mixed effects regression models were constructed as explained in **section 2.2.6.3**.

3.1.6.5 Linear mixed effects regression model (LM) for *Dichelobacter nodosus* load *(RStudio* v0.99.903, *R* v3.3.1)

Linear mixed effects regression models were used to investigate the effect of locomotion score, ID score, SFR score, foot region, whether a sample was taken in a routine or targeted sampling period, if a sample was culture positive, and who had extracted the DNA on *D. nodosus* load. Fixed effects were initially checked individually prior to running a multivariable linear mixed effects regression model accounting for sheep, foot, foot region, week and who had extracted the DNA. Linear mixed effects regression models were constructed using the 'lmer' function from the lme4 package in R (Bates *et al.*, 2015). The Akaike Information Criterion (AIC) was used to compare model fit and fixed effects were retained in the multivariable model if it resulted in a lower AIC.

3.1.6.6 Linear mixed effects regression model (LM) for *Dichelobacter nodosus* load of interdigital skin samples only by each treatment separately *(RStudio* v0.99.903, *R* v3.3.1)

Linear mixed effects regression models were used to investigate the effect of the three different treatments, FTA, PTA, and parenteral antibiotics only given for another health reason, on *D. nodosus* load. Each treatment was investigated individually accounting for sheep, foot and week. Linear mixed effects regression models were constructed as explained in **section 2.2.6.5**.

3.2 Key virulence factors, *aprB2*, *aprV2* and *fimA* of *Dichelobacter nodosus* and associations with the severity of footrot: Bristol study samples

3.2.1 Singleplex PCR (Dhungyel *et al.*, 2002) of individual swab samples containing mixed DNA to detect *Dichelobacter nodosus* serogroups present

In the current study presence of serogroups A – I were tested for. Each *D. nodosus* positive sample was tested for each of the nine serogroups using a singleplex PCR based on Dhungyel *et al.* (2002); nine separate reactions were run for each sample. Each reaction consisted of 2x MyTaq Red Mix (Bioline), 10µM forward primer, 10µM each reverse primer separately from A to I (Dhungyel *et al.*, 2002), 10mg/ml bovine serum albumin, 1µl DNA plus nuclease-free water resulting in a total reaction volume of 50µl. The reverse primer sequences and PCR program used are given in **Tables 3.1** and **3.2** respectively. Positive serogroup controls and a negative control (water) were included with each batch.

Table 3.4: *Dichelobacter nodosus fimA* PCR Primers (Dhungyel *et al.*, 2002).

Primer	Nucleotide sequence	Position in *fimA*	Product size (bp)
Forward	CCTTAATCGAACTCATGATTG	26 - 46	-
Reverse A	AGTTTCGCCTTCATTATATTT	421 - 441	415
Reverse B	CGGATCGCCAGCTTCTGTCTT	286 - 309	283
Reverse C	AGAAGTGCCTTTGCCGTATTC	331 - 351	325
Reverse D	TGCAACAATATTTCCCTCATC	325 - 345	319
Reverse E	CACTTTGGTATCGATCAACTTGG	367 - 389	363
Reverse F	ACTGATTTCGGCTAGACC	250 - 267	241
Reverse G	CTTAGGGGTAAGTCCTGCAAG	283 - 305	279
Reverse H	TGAGCAAGACCAAGTAGC	412 - 435	409
Reverse I	CGATGGGTCAGCATCTGGACC	194 - 215	189

Table 3.5: *Dichelobacter nodosus fimA* PCR program.

		Den.[a]	Ann.[b]	Ext.[c]	Den.[a]	Ann.[b]	Ext.[c]	
Temperature	94º	94º	60º	72º	94º	58º	72º	72º
Time	4:00	0:30	0:30	0:30	0:30	0:30	0:30	4:00
		x5 cycles			x25 cycles			

[a]Denaturation.
[b]Annealing.
[c]Extension.

3.2.2 Laboratory analysis of *aprB2* and *aprV2* qPCR on clinically healthy foot samples

Healthy samples only were analysed to see if *aprB2*, the benign form of *D. nodosus* linked with less severe footrot, could be detected. *D. nodosus* positive samples from feet with a locomotion score, ID score and SFR score of 0 were run through qPCR to detect for the presence of *aprB2* ("benign" *D. nodosus*) and *aprV2* ("virulent" *D. nodosus*). The *aprB2* and *aprV2* primer and probe sequences are given in **Table 3.3** and the qPCR standards in **Table 3.4**. Each reaction comprised of 2x Klearkall master mix (LGC Group), 10µM *aprB2V2* forward primer, 10µM *aprB2V2* reverse primer, 10µM *aprB2* Texas Red probe, 10µM *aprV2* FAM probe, 10mg/ml bovine serum albumin, 1µl DNA plus nuclease-free water resulting in a total reaction volume of 15µl. The thermal cycle profile consisted of a holding stage at 95º for 15 mins followed by 45 cycles of 95º for 3 secs and 60º for 30 secs.

Table 3.6: *Dichelobacter nodosus aprB2* and *aprV2* qPCR primers and probes (Frosth *et al.*, 2015).

Primer / Probe	Nucleotide sequence
Forward	5'-GAAGGCGACTGGTTTGATAACTG-3'
Reverse	5'-GAGCTGTCGCTTCTTTCTTTGC-3'
B2 probe	5'-TxRd-ATGCGGTGGTCGTCCT-BHQ2-3′
V2 probe	5′-6FAM-ATGCGGTGGTTATCCT-BHQ1-3′

6FAM: 6 carboxy-fluoroscein.
BHQ: Black Hole Quencher.
TxRd: Sulforhodamine 101-X.

Table 3.7: *Dichelobacter nodosus aprV2* and *aprB2* qPCR standards.

Dilution	Source of plasmid DNA	Initial conc. (ng/µl)	Volume of plasmid DNA (µl)	Volume of dilutent (µl)	Final volume (µl)	Final conc. (ng/µl)	Resulting copy number (*rpoD*/µl)
aprV2							
Stock	VCS1703A	1.15E-7	0.42	499.58	500	9.63E-11	N/A
10^6	Stock	9.63E-11	5.00	95.00	100	4.81E-12	1.00E+6
10^5	10^6	4.81E-12	10.00	90.00	100	4.81E-13	1.00E+5
10^4	10^5	4.81E-13	10.00	90.00	100	4.81E-14	1.00E+4
10^3	10^4	4.81E-14	10.00	90.00	100	4.81E-15	1.00E+3
10^2	10^3	4.81E-15	10.00	90.00	100	4.81E-16	1.00E+2
10^1	10^2	4.81E-16	10.00	90.00	100	4.81E-17	1.00E+1
aprB2							
Stock	C305	8.29E-8	0.58	499.42	500	9.63E-11	N/A
10^6	Stock	9.63E-11	5.00	95.00	100	4.81E-12	1.00E+6
10^5	10^6	4.81E-12	10.00	90.00	100	4.81E-13	1.00E+5
10^4	10^5	4.81E-13	10.00	90.00	100	4.81E-14	1.00E+4
10^3	10^4	4.81E-14	10.00	90.00	100	4.81E-15	1.00E+3
10^2	10^3	4.81E-15	10.00	90.00	100	4.81E-16	1.00E+2
10^1	10^2	4.81E-16	10.00	90.00	100	4.81E-17	1.00E+1

3.2.3 Statistical analysis

Plots were created using *MS Excel* v16.29. The Cochran-Mandel-Haenszel test (*Minitab Express* v1.5.0) was used to compare serogroup detection by swab DNA and isolate DNA analysis as some samples appeared in both categories. Kruskal-Wallis tests (*Minitab Express* v1.5.0) were used to investigate persistence of serogroups on feet over time. In all tests p values ≤ 0.05 were considered significant.

3.2.3.1 Binomial mixed effects regression model (BM) for detection of *Dichelobacter nodosus* by interdigital dermatitis and severe footrot score by serogroup *(RStudio* v0.99.903, *R* v3.3.1)

Binomial mixed effects regression models were used to investigate the effect of positive serogroup detection on ID score ≥ 2 and positive for SFR. Fixed effects were initially checked individually prior to running a multivariable binomial mixed effects regression model accounting for sheep, foot, foot region and week. Binomial mixed effects regression models were constructed as explained in **section 2.2.6.3**.

3.3 Key virulence factors and phylogenetic diversity of *Dichelobacter nodosus* and associations with the severity of footrot: On a single UK farm

3.3.1 Acquisition, preparation, and whole genome sequencing of *Dichelobacter nodosus* isolates from the Bristol study flock

D. nodosus isolates were sampled from ewes and lambs and DNA extracted as part of Smith *et al.* (2017). DNA was extracted from these isolates as follows. Each microfuge tube containing a *D. nodosus* isolate was centrifuged and any supernatant present was removed. DNA was then extracted from each *D. nodosus* isolate following the method in **section 2.2.3**. This resulted in DNA suspended in 50µl Elution Buffer. Some isolates had already had DNA extracted by the same method (Smith *et al.*, 2017). Each *D. nodosus* isolate DNA sample was whole genome sequenced using the Illumina HiSeq x10 and paired-end 150bp reads at the *Wellcome Sanger Institute*, Cambridge, UK using the Standard WGS Illumina Library protocols. All sequences were uploaded to the European Nucleotide Archive (ENA) under **study accession number ERP110974**. Annotated assemblies were produced and refined using the Pathogen Informatics pipeline at the *Wellcome Sanger Institute* (Page *et al.*, 2016).

The following mapping steps were done using the Pathogen Informatics pipeline at the *Wellcome Sanger Institute* (software available at https://github.com/sanger-pathogens/vr-codebase). Sample sequences were mapped to the single *D. nodosus* reference; Dichelobacter_nodosus_VCS1703A_GCF_000015345_1, **accession number CP000513** (Myers *et al.*, 2007) using *SMALT* v0.7.4 to produce a BAM file. *SMALT* was used to index the reference using a kmer size of 13 and a step size of 4 for paired-end reads. The reads were aligned using default parameters with the maximum insert size set at 3x the mean fragment size of the sequencing library. PCR duplicate reads were identified using *Picard* V1.92 and flagged as duplicates in the BAM file (software available at https://sourceforge.net/projects/smalt/). Variation detection was done using *samtools mpileup* v0.1.19 (Li *et al.*, 2009). All bases were filtered to remove those with uncertainty

in the base call. Variant quality had to be > 50 and mapping quality > 30. If not all reads gave the same base call, the allele frequency, as calculated by bcftools, was required to be either 0 for bases called the same as the reference, or 1 for bases called as a single nucleotide polymorphism (SNP). The majority base call was required to be present in at least 75% of reads mapping at the base, with a minimum mapping depth of four reads (at least two had to map to each strand). Strand bias, map bias and tail bias were required to be < 0.001. If these filters were not met the base call was termed uncertain. A pseudogenome was constructed by substituting the base call at each site in the reference genome. Any site called as uncertain was substituted with an N. With respect to the reference genome, insertions were ignored and deletions filled with N's in the pseudogenome to maintain the alignment and sequence length the same as the reference genome.

3.3.2 Quality control steps for *Dichelobacter nodosus* sequences

Sequences were compared with the *RefSeq* bacterial genome database (O'Leary, 2016), refseq-bacteria-k21-s1000.msh and distances from the *D. nodosus* isolates to the database genomes were calculated using *Mash* (Ondov *et al.*, 2016). *Mash* is an alignment-free method which estimates the sequence mutation rate between sequences directly from their MinHash sketches (locality-sensitive hashing technique) (Ondov *et al.*, 2016). Genomes that matched to the *D. nodosus* reference genome underwent quality control. Poor sequences were removed from the dataset if they met with one/more of the following criteria. Heterozygous SNPs refers to the number of mapped reads at each base of the reference. A high proportion of heterozygous SNPs may indicate mis-mapping of repeats or sample contamination. Depth of coverage refers to the minimum fragment length for assembly.

- Matched to anything other than *Dichelobacter nodosus* in the mash results
- Total assembly length < 1Mb or > 2Mb
- Number of heterozygous SNPS > 1000
- Depth of coverage < 20

"Duplicates" referred to multiple isolates obtained from the same foot of the same sheep on the same sampling day with the same acidic protease and serogroup identified from laboratory and genomic analysis. The isolate with the smallest number of contigs in the genome assembly was retained and any duplicates of the retained isolate were removed from analysis.

3.3.3 SNP analysis of *Dichelobacter nodosus* genomes

The phylogeny of the SNPs from the alignment were generated using the *Randomized Axelerated Maximum Likelihood (RAxML) v8.0.0* (Stamatakis, 2014) technique. Pairwise SNP count was used

to identify maximum SNP count. *Microreact* was used to visualise phylogenetic trees including the figures shown in this thesis (Argimon *et al.*, 2016). Where recombination prediction removal is indicated this was done using *Genealogies Unbiased By recomBinations In Nucleotide Sequences* (Gubbins) (Croucher *et al.*, 2015). Recombination predictions were visualised using *Phandango* (Hadfield *et al.*, 2018). The number of recombination events to mutation events (ρ/θ) and the probability that a site would be altered by recombination or mutation (r/m) were taken from the Gubbins output.

3.3.4 Clade and temporal signal analysis

Phylogenetic analysis of globally sourced *D. nodosus* isolates later described in **Chapter 5** was used to identify clades present within the Bristol study isolates. Each clade was then mapped against the genome of an isolate(s) not from the Bristol study isolates that was closest phylogenetically. Phylogenetic temporal signal correlations were calculated using *TempEst v1.5.1* showing root-to-tip linear regression of the genetic distances between isolates against the sampling time (Rambaut *et al.*, 2016). Indications of temporal signal from *TempEst* were investigated further using *BactDating: Bayesian inference of ancestral dates on bacterial phylogenetic trees* v1.0.5 (https://github.com/xavierdidelot/BactDating) (Didelot *et al.*, 2018) in *RStudio* v0.99.903, *R* v3.3.1. Markov chain Monte Carlo (MCMC) convergence was checked through parameter trace analysis. The significance of the temporal signal was calculated by comparing the deviance information criterion (DIC) of the resulting signal with the accurate sampling dates to the signal when sampling dates are forced to be equal (Didelot *et al.*, 2018).

3.3.5 Genomic analysis of *aprB2* and *aprV2* and the *fimA* gene to identify serogroups

Laboratory analysis of acidic protease present was not conducted. Serogroup identification by laboratory methods was determined in a previous study using a multiplex PCR (Smith *et al.*, 2017, Dhungyel *et al.*, 2002).

Acidic protease *V2* and *B2* are differentiated by a 2bp substitution of tyrosine (TAT) to arginine (CGT) respectively at position 661/662 (Riffkin *et al.*, 1995). The starting codons of the *fimA* gene as identified by Mattick *et al.* (1991) are shown in **Figure 4.1**, prior to the identification of serogroup M. A Python script including BLASTn (https://blast.ncbi.nlm.nih.gov/) was used to identify the percentage nucleotide identity in each isolate to the *aprB2* and *aprV2* reference sequences (**Table 4.1**) and *fimA* serogroup reference sequences (**Table 4.2**). The presence of acidic protease variant and serogroup of each isolate was determined to be the positively identified serogroup with the highest percentage nucleotide identity to a serogroup reference.

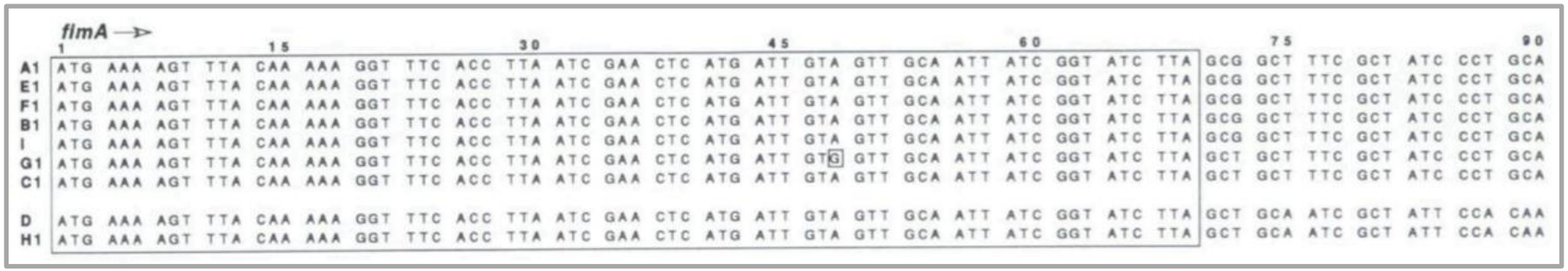


Figure 3.4: The first 23 codons of the *fimA* gene in *Dichelobacter nodosus* (*Bacteroides nodosus*) genome considered to be 100% conserved apart from a single base change in serogroup G (Mattick *et al.*, 1991).

Table 3.8: Acidic protease *B2* and *V2* reference sequences.

Acidic protease variant	GenBank sequence ID	Reference
B2	FN674446.1	(Kennan *et al.*, 2010)
V2	L38395.1	(Riffkin *et al.*, 1995)

Table 3.9: Serogroup and serotype reference sequences as determined by the *fimA* gene (Hobbs *et al.*, 1991, Mattick *et al.*, 1991).

Serogroup; serotypes	Isolate ID	GenBank sequence ID
A; A1, A2	VCS1001 (A1)	X52403.1
B; B1, B2, B3, B4	VCS1006 (B1)	X52404.1
C; C1, C2	VCS1008 (C1)	X52405.1
D	VCS1172	X52389.1
E; E1, E2	VCS1114 (E2)	X52407.1
F; F1, F2	VCS1017 (F1)	X52408.1
G; G1, G2	VCS1220 (G1)	X52409.1
H; H1, H2	VCS1215 (H1)	X52390.1
I	VCS1636	X52410.1

3.4 Key virulence factors and phylogenetic diversity of *Dichelobacter nodosus* and associations with the severity of footrot: Globally sourced isolates

3.4.1 Acquisition, preparation, and whole genome sequencing of globally sourced *Dichelobacter nodosus* isolates

D. nodosus isolates were sourced initially from published studies accessed through **study accession numbers PRJEB6348** (Kennan *et al.*, 2014) and **PRJNA386733** (Blanchard *et al.*, 2018). Additional *D. nodosus* isolates were sourced from the University of Warwick, Bristol Veterinary School, Swedish University of Agricultural Sciences, Norwegian Veterinary Institute and University of Veterinary Medicine Hannover. The majority of additional *D. nodosus* isolates were from sheep but also several sampled from cows and goats (shown in results). *D. nodosus* isolates needing whole genome sequencing were put through the same sequencing procedure, quality control steps and SNP analysis as described in **sections 4.2.1**, **4.2.2** and **4.2.3** where new sequences were processed under **study accession number ERP110974**. Where there was no suitable isolate to root a phylogenetic tree from, *TempEst* v1.5.1 'best fitting root' function was used giving the best indication of temporal signal (Rambaut *et al.*, 2016). Recombination predictions were visualised using *Phandango* (Hadfield *et al.*, 2018).

3.4.2 Laboratory analysis of *aprB2* and *aprV2* and the *fimA* gene to identify serogroups

For all isolates not included in the Bristol study, acidic protease and serogroup detection information was collected from the isolate sources wherever possible.

3.4.3 Genomic analysis of *aprB2* and *aprV2* and the *fimA* gene to identify serogroups

As described in **section 4.2.5**.

4 Results

4.1 Detection of *Dichelobacter nodosus* and associations between load and the severity of footrot: Bristol Study flock samples

From the Bristol study flock of 99 ewes, eight died during the study so their data were removed from all analyses in the current study.

4.1.1 Levels of lameness in the subset of 25 ewes compared with the remaining 66 ewes

When analysing data from the Bristol study flock, a case of lameness was identified if a ewe had a locomotion score > 2. A new case of lameness was subsequently identified if a ewe had a locomotion score of 0 between treatments. In the study flock of 91 ewes, there was no significant difference in the median number of cases of lameness per ewe between the FTA group and PTA group, $p > 0.05$ Kruskal-Wallis test (**Table 2.4**). There was also no significant difference in the median number of ewes with their respective number of cases of lameness in the subset of 25 ewes or the remaining 66 ewes, $p > 0.05$ Kruskal-Wallis test (**Table 2.4**). **Figure 2.4** shows the number of cases of lameness for the subset of 25 ewes and remaining 66 ewes.

Table 4.1: Number of cases of lameness per ewe in the subset of 25 ewes and the remaining 66 ewes in the Bristol study flock by treatment group.

	SUBSET OF 25 EWES			REMAINING 66 EWES OF THE STUDY FLOCK		
No. of cases of lameness	**No. of ewes (%)**	**No. of ewes from FTA treatment group**	**No. of ewes from PTA treatment group**	**No. of ewes (%)**	**No. of ewes from FTA treatment group**	**No. of ewes from PTA treatment group**
0	3 (12)	2	1	15 (23)	9	6
1	6 (24)	4	2	17 (26)	6	11
2	9 (36)	3	6	14 (21)	10	4
3	2 (8)	0	2	9 (14)	3	6
4	2 (8)	2	0	6 (9)	4	2
5	0	0	0	3 (5)	1	2
6	1 (4)	1	0	2 (3)	1	1
7	2 (8)	0	2	0	0	0
TOTAL	25	12	13	66	34	32

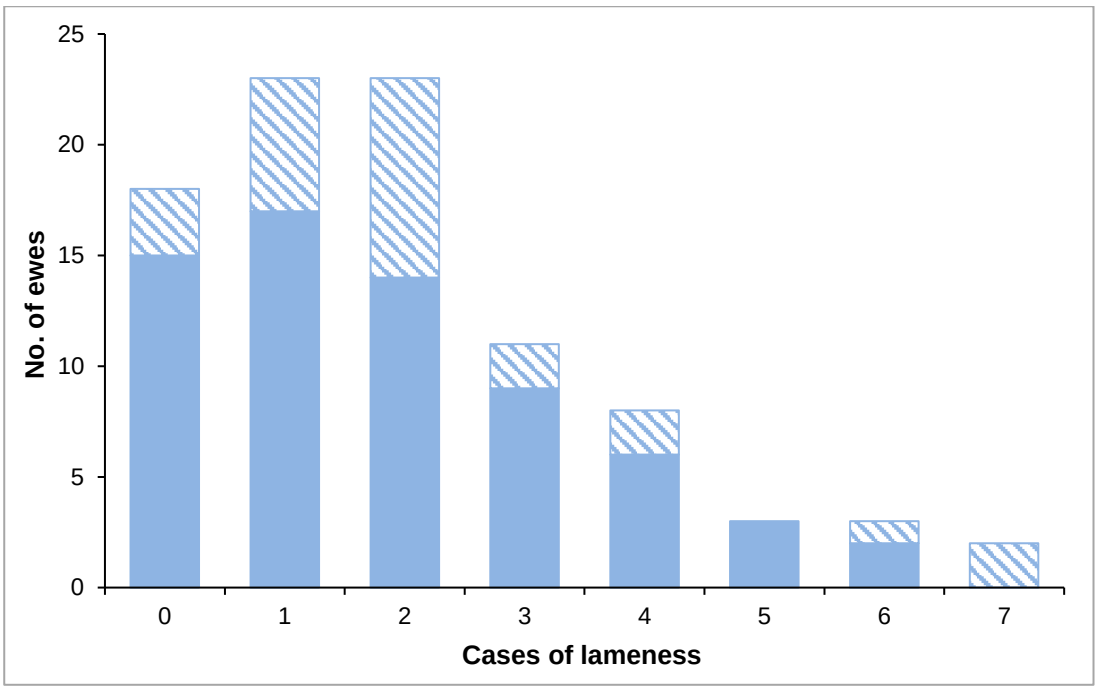


Figure 4.1: Number of ewes by number of cases of lameness in the subset of 25 ewes (*blue stripe*) and the remaining 66 ewes of the Bristol study flock (*blue*) over the study period indicating a skewed distribution.

4.1.2 Proportion of locomotion, interdigital dermatitis and severe footrot scores recorded for the subset of 25 ewes in routine and targeted sampling weeks

Locomotion scores 0 – 4 were observed in routine and targeted sampling weeks, however, locomotion and footrot lesion scores did not always correlate. For example, in routine sampling weeks, 50% (266) of ewes' feet with locomotion score 0 had a positive ID and/or positive SFR score; 31% (168) positive for ID only, 10% (55) positive for SFR only, and 8% (43) positive for ID and SFR. In addition, 34% (11) of samples taken in routine sampling weeks from ewes with a more severe locomotion score requiring treatment (3 or 4) had ID and SFR scores of 0. In targeted sampling weeks, 60% (53) of ewes' feet with a more severe locomotion score requiring treatment (3 or 4) had ID and SFR scores of 0. One ewe from the subset of 25 had a locomotion score of 0 throughout the study. Another ewe from the subset of 25 had only one occurrence of a positive locomotion score. But both ewes had multiple occurrences of positive ID (highest at 4) and SFR scores during routine recordings.

Table 4.2: Number of samples taken in routine and targeted sampling periods by locomotion score for the subset of 25 ewes.

Locomotion score	No. of samples taken	No. of samples taken during routine sampling weeks	No. of samples taken during targeting sampling weeks
0	635	537	98
1	8	2	6
2	32	16	16
3	108	26	82
4	12	6	6
TOTAL	795	587	208

4.1.3 Treatment for footrot and recovery from lameness and lesions in the Bristol study flock of 91 ewes

Of the 91 ewes, 77 received at least one treatment for footrot during the study and 65 of these had cases with complete follow up data for the two weeks after treatment. Recovery was assessed in terms of whether positive scores for locomotion and positive scores for footrot reduced or not two weeks after treatment. The number of cases of lameness where positive lameness scores and positive footrot lesion scores reduced or not by type of treatment is given in **Table 2.6**. There was no difference between the two designated treatments and positive locomotion score reducing two weeks after treatment, $p > 0.05$ BM (**Table 2.7**). However, a reduction in positive footrot lesion scores occurred in more cases by treatment with FTS than with PTS, $p = 0.008$ BM (**Table 2.7**). The number of treatments given each week for the Bristol flock of 91 ewes is given in **Appendix 2**.

Table 4.3: Number and percentage of cases where positive lameness scores and positive footrot scores were reduced or not two weeks after treatment.

		FOOT TRIM + TOPICAL ANTIBIOTIC FOOT SPRAY	PARENTERAL ANTIBIOTICS + TOPICAL ANTIBIOTIC FOOT SPRAY
LOCOMOTION SCORE	**Reduced**	62 (88.6)	72 (85.7)
	Not reduced	8 (11.4)	12 (14.3)
FOOTROT SCORE(S)	**Reduced**	42 (77.8)	28 (52.8)
	Not reduced	12 (22.2)	25 (47.2)

Table 4.4: Separate binomial mixed effects regression models of the two treatments on lameness and lesion reduction.

Predictors		LAMENESS REDUCED					
		No.	**%**	**Odds R.**	**CI**	**CS**	**p value**
Treatment (FTS)		62	88.6	*Baseline*			
Treatment (PTS)		72	85.7	0.52	0.05 – 5.01	-0.56	0.574
Random effects							
Variance (σ^2)	3.29						
Sheep ID	18.54						
ICC	0.85						
No. Sheep ID	65						
Observations	154						
Marginal R^2	0.005						
Conditional R^2	0.850						
Predictors		FOOTROT LESIONS REDUCED					
		No.	**%**	**Odds R.**	**CI**	**CS**	**p value**
Treatment (FTS)		42	77.8	*Baseline*			
Treatment (PTS)		28	52.8	0.32	0.14 – 0.74	-2.66	**0.008**
Random effects							
Variance (σ^2)	3.29						
Sheep ID	0.00						
ICC	-						
No. Sheep ID	54						
Observations	107						
Marginal R^2	0.091						
Conditional R^2	NA						

CI: Confidence intervals.
CS: Coefficient statistic.
Odds R.: Odds Ratios.

4.1.4 Overview of *Dichelobacter nodosus* detected on samples in the subset of 25 ewes

A total of 845 interdigital skin swabs and 78 non-interdigital lesions swabs were processed from the selected feet of the 25 ewes with a summary of statistics shown in **Table 2.8**. Interdigital skin sample ID scores ranged from 0 to 4. There were 3 samples with SFR score 2, the remainder were either SFR score 0 or 1. All non-interdigital lesion samples had a SFR score of 1 and ID scores ranged from 0 to 4.

In these analyses, as there were only three occurrences of a SFR score > 1, samples with a SFR score ≥ 1 were coded as 1 for presence of SFR.

Table 4.5: Number of interdigital skin and non-interdigital lesion swabs from the sub-set of two feet of 25 ewes.

	Interdigital skin	Non-interdigital lesion
No.	845	78
Mean	17	2
Median	16	1
Range	13 - 26	0 - 8

A total of 578 (68.4%) of the 845 interdigital skin swabs analysed were positive for *D. nodosus* and 48 (61.5%) of the 78 non-interdigital lesion samples analysed were positive for *D. nodosus*. There was no significant difference in *D. nodosus* detection between interdigital skin samples and non-interdigital lesion samples, $p > 0.05$ BM (**Table 2.13**, pg. 41). There was also no significant difference in *D. nodosus* detection between the three extractors, $p > 0.05$ BM (**Table 2.9**).

Table 4.6: Number and percentage of samples processed through extraction and qPCR by each extractor.

		INTERDIGITAL SKIN SAMPLES		NON-INTERDIGITAL LESION SAMPLES	
Extractor*	**Extraction period**	**No. of samples**	**No. (%) of samples positive for *D. nodosus***	**No. of samples**	**No. (%) of samples positive for *D. nodosus***
1	2010/2011	64	48 (75)	3	2 (66.7)
2	2010/2011	192	146 (76.0)	14	9 (64.3)
3	2016	589	384 (65.2)	61	37 (60.7)

*Extractors 1 & 2 are authors of Smith *et al.*, 2014 and extractor 3 is the author of this thesis.

4.1.5 *Dichelobacter nodosus* detection by locomotion, interdigital dermatitis and severe footrot score

*Multivariable binomial mixed effects regression model results are given in **Table 2.13** with univariable results given in **Appendix 3**.*

Samples from feet with a locomotion score of 3 had a higher proportion of samples positive for *D. nodosus* than those with locomotion score 0, $p < 0.002$ BM (**Table 2.13**). Proportion of interdigital skin sample positive for *D. nodosus* decreased through locomotion scores 0, 1 and 2 then increased to locomotion scores 3 and 4 (**Table 2.10**). As locomotion score increased the proportion of non-interdigital lesion samples positive for *D. nodosus* increased (**Table 2.10**). All samples with a locomotion score of 4 were positive for *D. nodosus*.

Table 4.7: Number and percentage of interdigital skin and non-interdigital lesion samples positive for *D. nodosus* by locomotion score.

Locomotion score	No. of samples	No. (%) of samples positive for *D. nodosus*
Interdigital skin samples		
0	635	415 (65.4)
1	8	5 (62.5)
2	32	11 (34.4)
3	108	86 (79.6)
4	12	12 (100)
Non-interdigital lesion samples		
0	30	13 (43.3)
1	0	0
2	3	2 (66.7)
3	41	29 (70.7)
4	4	4 (100)

Samples with an ID score of 2, 3 or 4 had a higher proportion of samples positive for *D. nodosus* than those with an ID score of 0, p < 0.002, < 0.003 and < 0.005 respectively BM (**Table 2.13**). As ID score increased the proportion of interdigital skin samples positive for *D. nodosus* increased, with the exception of samples with an ID score of 4 and positive for SFR where the proportion positive for *D. nodosus* decreased (**Table 2.11**). In cases of both SFR0 and SFR1 the greatest increase in the proportion of interdigital skin samples positive for *D. nodosus* was from ID1 to ID2 (increase of 23.7% ± 3.3%), compared to a mean increase of only 2.9% ± 4.9% between the other ID score increases. As ID score increased, the proportion of non-interdigital lesion samples positive for *D. nodosus* increased up to ID4 where the proportion positive decreased (**Table 2.12**).

Table 4.8: Number and percentage of interdigital skin samples positive for *D. nodosus* by ID score and whether SFR was present.

	SFR0		SFR1		REGARDLESS OF SFR SCORE	
ID score	**No. of samples**	**No. (%) of samples positive for *D. nodosus***	**No. of samples**	**No. (%) of samples positive for *D. nodosus***	**No. of samples**	**No. (%) of samples positive for *D. nodosus***
0	438	265 (60.5)	83	50 (60.2)	521	315 (60.5)
1	143	102 (71.3)	25	16 (64.0)	168	118 (70.2)
2	41	38 (92.7)	20	18 (90.0)	61	56 (91.8)
3	26	24 (92.3)	12	11 (91.7)	38	35 (92.1)
4	40	39 (97.5)	17	15 (88.2)	57	54 (94.7)

SFR0: Negative for severe footrot.
SFR1: Positive for severe footrot.

Table 4.9: Number and percentage of non-interdigital lesion samples positive for *D. nodosus* by ID score (all positive for SFR).

ID Score	Total no. of samples	No. (%) of samples positive for *D. nodosus*
0	41	21 (51.2)
1	14	8 (57.1)
2	10	8 (80.0)
3	5	5 (100)
4	8	6 (75.0)

Table 4.10: Multivariable binomial mixed effects regression model of disease severity and sampling characteristics on *D. nodosus* detection.

			D. nodosus DETECTION			
Predictors	**No.**	**%**	**Odds R.**	**CI**	**CS**	**p value**
Foot region (Non-interdigital lesion)	78	8.5	*Baseline*			
Foot region (Interdigital skin)	845	91.5	2.42	0.93 – 6.31	1.80	0.072
Routine or Targeted (Routine)	665	72.0	*Baseline*			
Routine or Targeted (Targeted)	258	28.0	0.53	0.19 – 1.45	-1.24	0.216
Locomotion score 0	665	76.2	*Baseline*			
Locomotion score 1	8	0.9	2.65	0.44 – 16.04	1.06	0.290
Locomotion score 2	33	3.8	0.53	0.19 – 1.51	-1.18	0.237
Locomotion score 3	151	17.3	2.85	1.42 – 5.74	2.94	**0.003**
Locomotion score 4**	16	1.8	-	-	-	-
ID score 0	562	60.9	*Baseline*			
ID score 1	182	19.7	1.43	0.84 – 2.42	1.33	0.260
ID score 2	71	7.7	5.36	1.94 – 14.80	3.24	**0.001**
ID score 3	43	4.7	9.89	2.48 – 39.47	3.24	**0.001**
ID score 4	65	7.0	9.60	2.77 – 33.19	3.57	**< 0.001**
SFR score 0	688	74.5	*Baseline*			
SFR score 1	235	25.5	0.75	0.44 – 1.31	-1.00	0.315
Culture negative	875	94.8	*Baseline*			
Culture positive**	48	5.2	-	-	-	-

Random effects	
Variance (σ^2)	3.29
Foot region : (Foot : Sheep ID)	0.64
Foot : Sheep ID	0.00
Sheep ID	0.32
Week	1.63
Extractor	0.01
No. Foot region	2
No. Foot	4
No. Sheep ID	25
No. Week	42
No. Extractor	3
Observations	873
Marginal R^2	0.858
Conditional R^2	NA

CI: Confidence intervals.
CS: Coefficient statistic.
Odds R.: Odds Ratios.
**All samples were *D. nodosus* positive or only one sample was negative.

Log_{10}(*D. nodosus* load +1) of *D. nodosus* positive interdigital skin samples and non-interdigital lesion samples do not follow normal distributions, Anderson-Darling normality test $p < 0.005$ (**Figure 2.5**) and $p = 0.0278$ (**Figure 2.6**) respectively.

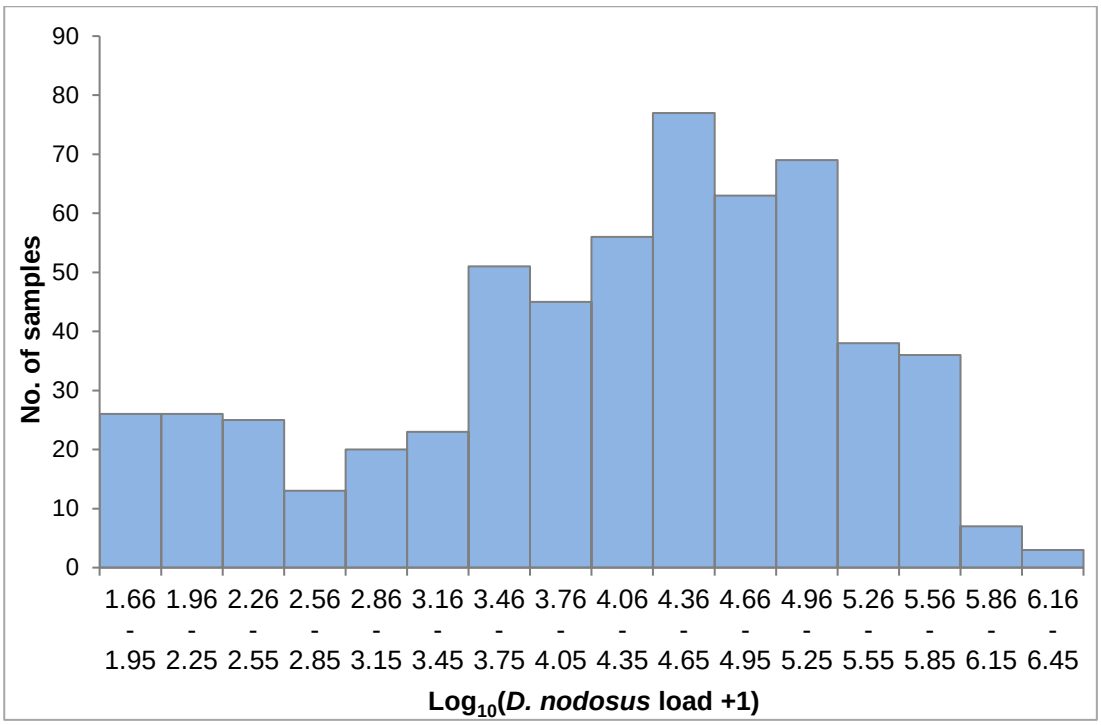


Figure 4.2: Number of *D. nodosus* positive interdigital skin samples by $\log_{10}$(*D. nodosus* load +1) indicating a slightly bimodal distribution, Anderson-Darling normality test.

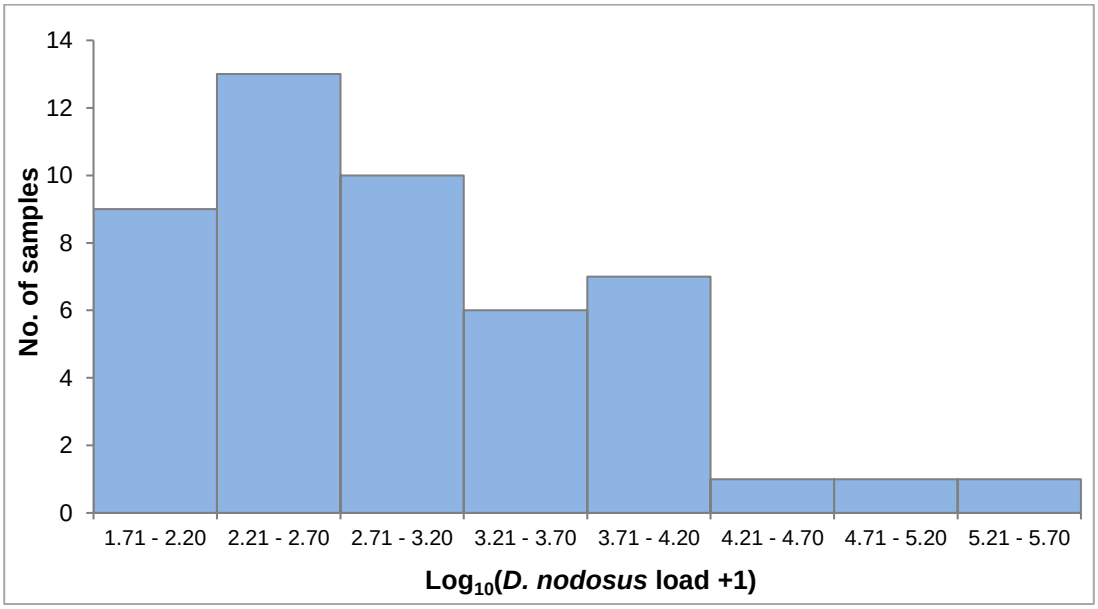


Figure 4.3: Number of *D. nodosus* positive non-interdigital lesion samples by $\log_{10}$(*D. nodosus* load +1) indicating a slightly skewed distribution, Anderson-Darling normality test.

4.1.5.1 *Dichelobacter nodosus* load by locomotion, interdigital dermatitis and severe footrot score

Multivariable linear mixed effects regression model results are given in ***Table 2.18*** *with univariable results given in* ***Appendix 4****.*

Mean $\log_{10}$(*D. nodosus* load +1) of interdigital skin samples was significantly higher than non-interdigital lesion samples, $p < 0.001$ LM (**Tables 2.18**). The mean $\log_{10}$(*D. nodosus* load +1) ± standard error of all and of *D. nodosus* positive only interdigital skin samples was 2.85 ± 0.07 and 4.16 ± 0.05 respectively. The mean $\log_{10}$(*D. nodosus* load +1) ± standard error of all and of *D. nodosus* positive only non-interdigital lesion samples was 1.81 ± 0.18 and 2.94 ± 0.13 respectively. There was no significant difference in mean $\log_{10}$(*D. nodosus* load +1) between the three extractors, $p > 0.05$ LM (**Table 2.18**).

Significant differences in *D. nodosus* load were found for locomotion scores and ID scores but not SFR scores. As locomotion score increased from 0 to 2, mean $\log_{10}$(*D. nodosus* load +1) decreased then sharply increased to locomotion scores 3 and 4 (**Table 2.14**). Mean $\log_{10}$(*D. nodosus* load +1) was significantly higher on feet from sheep with locomotion scores 3 and 4 than locomotion score 0, $p < 0.001$ and $p = 0.007$ respectively LM (**Table 2.18**). As ID score increased, mean $\log_{10}$(*D. nodosus* load +1) increased (**Tables 2.15** and **2.16**). Mean $\log_{10}$(*D. nodosus* load +1) in feet with ID scores 1 ($p = 0.016$), 2 ($p < 0.001$), 3 ($p < 0.001$) and 4 ($p < 0.001$) were significantly higher than feet with ID score 0, LM (**Table 2.18**). As with the proportion of samples positive for *D. nodosus*, the greatest increase in mean $\log_{10}$(*D. nodosus* load +1) regardless of SFR score was from ID1 to ID2 (**Table 2.17**). The increase here was more than 3 times greater than the other ID score increases.

Table 4.11: Mean $\log_{10}$(*D. nodosus* load +1) ± standard error of interdigital skin samples (795 records) and non-interdigital lesion samples (78 records) by locomotion score.

Locomotion Score	ALL SAMPLES		*D. nodosus* POSITIVE SAMPLES ONLY	
	No.	**Mean ± s.e.**	**No. (%*)**	**Mean ± s.e.**
Interdigital skin samples				
0	635	2.70 ± 0.09	415 (65.4)	4.13 ± 0.05
1	8	2.23 ± 0.71	5 (62.5)	3.57 ± 0.47
2	32	1.41 ± 0.29	11 (34.4)	3.87 ± 0.54
3	108	3.41 ± 0.20	86 (79.6)	4.34 ± 0.12
4	12	4.15 ± 0.39	12 (100)	4.15 ± 0.39
Non-interdigital lesion samples				
0	30	1.25 ± 0.30	13 (43.3)	3.07 ± 0.24
1	0	-	0	-
2	3	1.49 ± 0.79	2 (66.7)	2.24 ± 0.47
3	41	2.00 ± 0.24	29 (70.7)	2.83 ± 0.17
4	4	3.62 ± 0.25	4 (100)	3.62 ± 0.25

Table 4.12: Mean $\log_{10}$(*D. nodosus* load +1) ± standard error of interdigital skin samples by ID and SFR score.

ID score	SFR0				SFR1			
	ALL SAMPLES		*D. nodosus* POSITIVE SAMPLES ONLY		ALL SAMPLES		*D. nodosus* POSITIVE SAMPLES ONLY	
	No.	**Mean ± s.e.**	**No. (%*)**	**Mean ± s.e.**	**No.**	**Mean ± s.e.**	**No. (%*)**	**Mean ± s.e.**
0	438	2.32 ± 0.10	265 (60.5)	3.84 ± 0.07	83	2.50 ± 0.24	50 (60.2)	4.14 ± 0.15
1	143	2.99 ± 0.17	102 (71.3)	4.19 ± 0.10	25	2.59 ± 0.43	16 (64)	4.05 ± 0.26
2	41	4.24 ± 0.23	38 (92.7)	4.57 ± 0.13	20	4.17 ± 0.37	18 (90)	4.63 ± 0.21
3	26	4.35 ± 0.32	24 (92.3)	4.71 ± 0.21	12	4.28 ± 0.46	11 (91.7)	4.67 ± 0.26
4	40	4.95 ± 0.18	39 (97.5)	5.08 ± 0.13	17	4.14 ± 0.44	15 (88.2)	4.69 ± 0.25

*Percentage of all *D. nodosus* positive samples for each ID lesion score.

Table 4.13: Mean log_{10}(*D. nodosus* load +1) ± standard error of interdigital skin samples (845) and non-interdigital lesion samples (78) by ID score.

ID score	ALL SAMPLES		*D. nodosus* POSITIVE SAMPLES ONLY	
	No.	**Mean ± s.e.**	**No. (%*)**	**Mean ± s.e.**
*Interdigital skin samples***				
0	521	2.35 ± 0.09	315 (60.5)	3.89 ± 0.06
1	168	2.93 ± 0.16	118 (70.2)	4.17 ± 0.09
2	61	4.21 ± 0.19	56 (91.8)	4.59 ± 0.11
3	38	4.33 ± 0.26	35 (92.1)	4.70 ± 0.17
4	57	4.71 ± 0.19	54 (94.7)	4.97 ± 0.12
*Non-interdigital lesion samples****				
0	41	0.53 ± 0.14	21 (51.2)	2.68 ± 0.14
1	14	2.49 ± 0.05	8 (57.1)	2.48 ± 0.21
2	10	3.09 ± 0.07	8 (80.0)	3.37 ± 0.39
3	5	3.70 ± 0.06	5 (100)	3.35 ± 0.37
4	8	4.39 ± 0.17	6 (75.0)	3.51 ± 0.48

*Percentage of all *D. nodosus* positive samples for each ID lesion score.
**Regardless of SFR score.
***All SFR1.

Table 4.14: Mean increase in log_{10}(*D. nodosus* load +1) ± standard deviation of interdigital skin samples and standard deviation per ID score regardless of SFR score.

ID score increase	Mean increase in log_{10}(*D. nodosus* load +1) ± s.d. for all samples	Mean increase in log_{10}(*D. nodosus* load +1) ± s.d. for *D. nodosus* positive sample only
1 – 2	1.28	0.76
0 – 1, 2 – 3, 3 – 4	0.36 ± 0.23	0.22 ± 0.10

Table 4.15: Multivariable linear mixed effects regression model of disease severity and sampling characteristics on log_{10}(*D. nodosus* load +1).

			LOG$_{10}$(*D. nodosus* LOAD +1)		
Predictors	**No.**	**%**	**Estimates**	**CI**	**p value**
Foot region (Non-interdigital lesion)	78	8.5	*Baseline*		
Foot region (Interdigital skin)	845	91.5	1.20	0.63 – 1.76	**< 0.001**
Routine or Targeted (Routine)	665	72.0	*Baseline*		
Routine or Targeted (Targeted)	258	28.0	-0.74	-1.30 – -0.18	**0.010****
Locomotion score 0	665	76.2	*Baseline*		
Locomotion score 1	8	0.9	0.88	-0.35 – 2.12	0.162
Locomotion score 2	33	3.8	-0.37	-1.03 – 0.29	0.267
Locomotion score 3	151	17.3	0.71	0.31 – 1.11	**0.001**
Locomotion score 4	16	1.8	1.51	0.59 – 2.43	**0.001**
ID score 0	562	60.9	*Baseline*		
ID score 1	182	19.7	0.44	0.12 – 0.77	**0.007**
ID score 2	71	7.7	1.25	0.78 – 1.71	**< 0.001**
ID score 3	43	4.7	1.63	1.04 – 2.22	**< 0.001**
ID score 4	65	7.0	1.97	1.38 – 2.57	**< 0.001**
SFR score 0	688	74.5	*Baseline*		
SFR score 1	235	25.5	-0.13	-0.45 – 0.19	0.433
Culture negative	875	94.8	*Baseline*		
Culture positive	48	5.2	1.30	0.74 – 1.86	**< 0.001**

Random effects	
Variance (σ^2)	2.63
Foot region : (Foot : Sheep ID)	0.33
Foot : Sheep ID	0.00
Sheep ID	0.34
Week	0.50
Extractor	0.00
No. Foot region	2
No. Foot	4
No. Sheep ID	25
No. Week	42
No. Extractor	3
Observations	873
Marginal R^2	0.240
Conditional R^2	NA

CI: Confidence intervals.
**Significantly associated with locomotion scores 3 and 4.

4.1.5.2 Comparison of *Dichelobacter nodosus* load of interdigital skin samples by interdigital dermatitis severity of the 25 ewes taken in routine and targeted sampling periods

There was no significant difference in mean log_{10}(*D. nodosus* load +1) between samples taken in routine or targeted sampling periods tested as a single factor, $p > 0.05$ LM (**Appendix 4**). The was a significant difference between sampling periods when included in the multivariable model in **Table**

2.18 however, mean $\log_{10}$(*D. nodosus* load +1) in targeted sampling weeks was significantly associated with locomotion scores 3 and 4, p values ≤ 0.003 LM.

4.1.5.3 Comparison of *Dichelobacter nodosus* load between interdigital skin samples that were *Dichelobacter nodosus* culture positive or negative from the subset of 25 ewes

D. nodosus was detected by quantitative PCR in all 47 (6%) *D. nodosus* culture positive interdigital skin samples from the 25 ewes. The mean $\log_{10}$(*D. nodosus* load +1) ± standard error for *D. nodosus* qPCR positive and culture positive interdigital skin samples was significantly higher ($p < 0.001$, LM **Table 2.18**) than *D. nodosus* qPCR positive but culture negative interdigital skin samples; 4.63 ± 0.11 and 4.12 ± 0.05 respectively. The minimum and maximum $\log_{10}$(*D. nodosus* load +1) for *D. nodosus* qPCR positive and culture positive interdigital skin samples, ranged from 3.16 to 6.00, or 1450 to > 996,000 *D. nodosus* cells, with one outlier of 2.21, or < 170 *D. nodosus* cells (**Figure 2.7**). The minimum and maximum $\log_{10}$(*D. nodosus* load +1) for *D. nodosus* qPCR positive but culture negative interdigital skin samples ranged from 1.68 to 6.43, or < 50 to > 2.6 million *D. nodosus* cells (**Figure 2.7**). For the 47 *D. nodosus* qPCR positive and culture positive interdigital skin samples, there was one (2%) outlier with a $\log_{10}$(*D. nodosus* load +1) value below the minimum value of 3.16. Whereas the $\log_{10}$(*D. nodosus* load +1) of 109 of the 531 (21%) *D. nodosus* qPCR positive but culture negative interdigital skin samples was below 3.16. *D. nodosus* cells present below the count of 1450 were not detected through culturing.

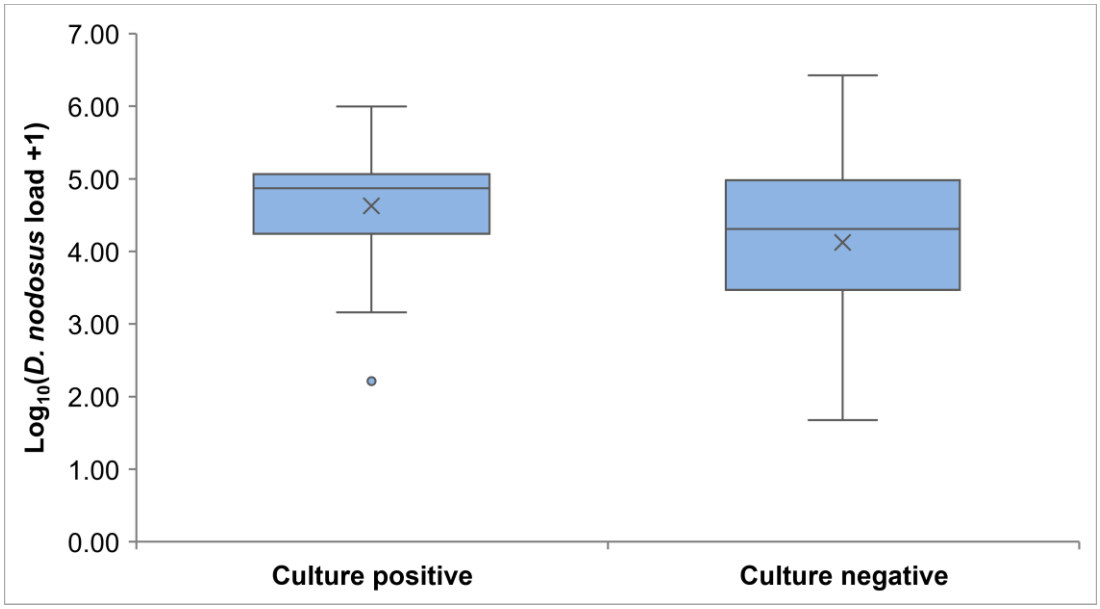


Figure 4.4: Box and whisker plot of median, mean (x), quartiles, range and outliers (o) for *D. nodosus* qPCR positive interdigital skin samples that were *D. nodosus* culture positive (47) and *D. nodosus* culture negative (531).

4.1.6 Effect of treatment on *Dichelobacter nodosus* load detected on interdigital skin samples of the 25 ewes

Mean $\log_{10}$(*D. nodosus* load +1) by treatment and time point is given in **Table 2.19**. Mean $\log_{10}$(*D. nodosus* load +1) of samples one week post-treatment with FTA, two weeks post-treatment with FTA, and samples from sheep in the FTA treatment group outside of these FTA treatment associated weeks (deemed healthy), were significantly lower than mean $\log_{10}$(*D. nodosus* load +1) of samples on the day of treatment with FTA, p values < 0.001, < 0.001 and 0.025 respectively, LM (**Table 2.20**). Mean $\log_{10}$(*D. nodosus* load +1) of samples one week post-treatment with PTA, two weeks post-treatment with PTA, and samples from sheep in the PTA treatment group outside of these PTA treatment associated weeks (deemed healthy) were significantly lower than mean $\log_{10}$(*D. nodosus* load +1) of samples on the day of treatment with PTA, p values < 0.001, LM (**Table 2.20**). This pattern was not observed for parenteral antibiotics given for another reason (no topical antibiotic foot spray was used). There was no significant difference between mean $\log_{10}$(*D. nodosus* load +1) of samples on the day of treatment with parenteral antibiotics only and one week post-treatment or two weeks post-treatment with parenteral antibiotics only, p > 0.05 LM (**Table 2.18**). The full range of ID scores 0 – 4 and SFR scores (0 – 1) recorded in this study were present in samples from both the FTA and PTA treatment groups. Samples where parenteral antibiotics only was given had ID scores of 0, 1 and 4 observed and SFR scores of 0 and 1 observed.

Table 4.16: Mean $\log_{10}$(*D. nodosus* load +1) ± standard error by treatment and time point.

Treatment	Time point	No. samples	Mean $\log_{10}$(*D. nodosus* load +1) ± s.e.
FTA	No treatment	120	2.23 ± 0.20
	On the day of treatment	28	4.16 ± 0.30
	1 week post-treatment	28	1.43 ± 0.30
	2 weeks post-treatment	28	2.12 ± 0.32
PTA	No treatment	102	2.70 ± 0.20
	On the day of treatment	34	3.80 ± 0.27
	1 week post-treatment	34	0.88 ± 0.27
	2 weeks post-treatment	34	1.42 ± 0.34
PA no TA	No treatment	-	-
	On the day of treatment	6	3.19 ± 0.48
	1 week post-treatment	6	3.68 ± 0.86
	2 weeks post-treatment	6	3.07 ± 1.00

FTA: Foot trim plus topical antibiotic foot spray.
No treatment: Mean $\log_{10}$(*D. nodosus* load +1) was calculated from samples outside of the treatment associated weeks.
PTA: Parenteral antibiotics plus topical antibiotic foot spray.
PA no TA: Parenteral antibiotics and no topical antibiotics used.

Further illustrated in **Figure 2.8**, the mean log_{10}(*D. nodosus* load +1) on the day of treatment being given, one week after treatment and two weeks after treatment were very similar for samples from ewes that received FTA and those that received PTA. Mean log_{10}(*D. nodosus* load +1) dropped by 66% for FTA and 77% for PTA one week after treatment followed by an increase of 48% for FTA and 61% for PTA two weeks after treatment. In the case of ewes treated with parenteral antibiotics for another reason and no topical antibiotics (six occasions) a decrease in mean log_{10}(*D. nodosus* load +1) one week after treatment was not observed.

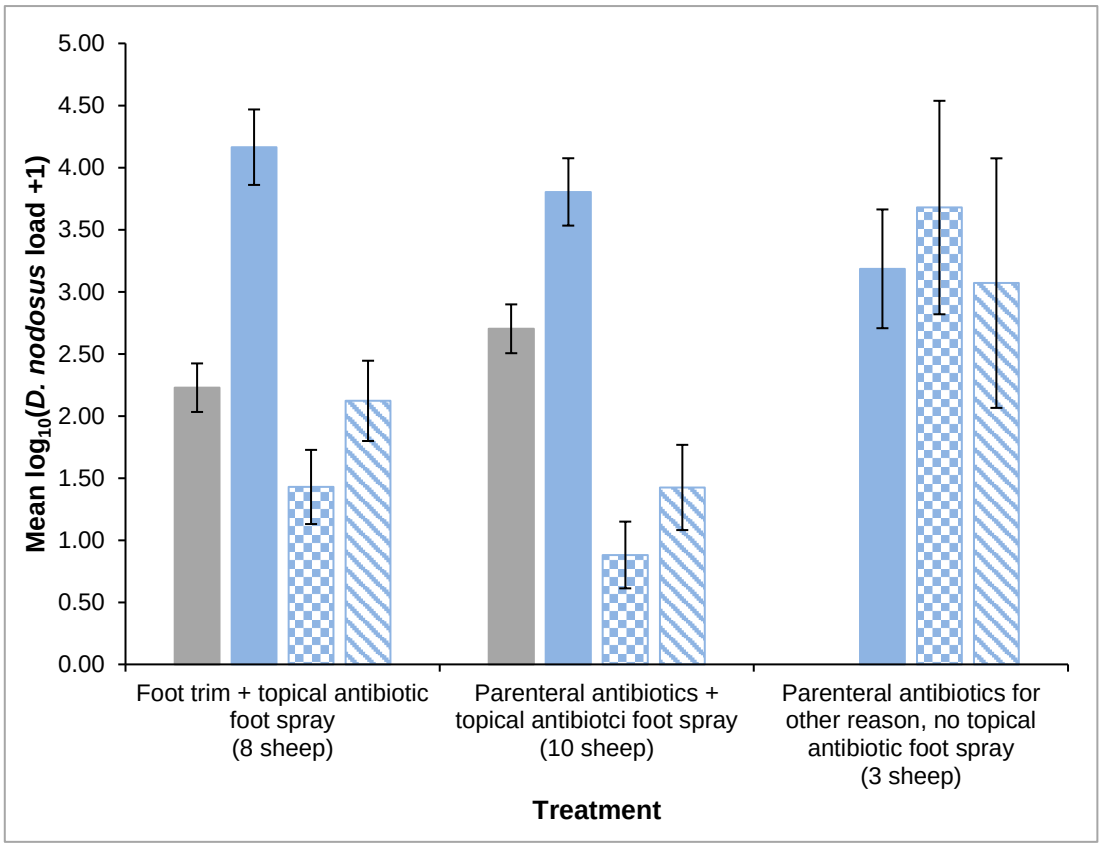


Figure 4.5: Mean log_{10}(*D. nodosus* load +1) for the three different treatment combinations given.

Blue: mean log_{10}(*D. nodosus* load +1) ± standard error on the day of treatment being given, *blue checked*: mean log_{10}(*D. nodosus* load +1) ± standard error one week after treatment was given, *blue stripe*: mean log_{10}(*D. nodosus* load +1) ± standard error and two weeks after treatment was given for the three treatments, *grey*: mean log_{10}(*D. nodosus* load +1) ± standard error of samples outside of the treatment associated weeks.

Table 4.17: Three separate univariable linear mixed effects regression models of the three treatment associated time points; on the day of treatment (0), one week after treatment (1), two weeks after treatment (2) and of foot samples from feet of sheep outside of these treatment associated weeks (99), for the three treatments given on mean log_{10}(*D. nodosus* load +1).

FTA LOG$_{10}$(*D. nodosus* LOAD +1)					PTA LOG$_{10}$(*D. nodosus* LOAD +1)					PA no TA LOG$_{10}$(*D. nodosus* LOAD +1)				
Predictors	**No.**	**Estimates**	**CI**	**p value**	**Predictors**	**No.**	**Estimates**	**CI**	**p value**	**Predictors**	**No.**	**Estimates**	**CI**	**p value**
FTA0	28	*Baseline*			PTA0	34	*Baseline*			PA no TA0	6	*Baseline*		
FTA1	28	-2.51	-3.42 – -1.60	**<0.001**	PTA1	34	-2.67	-3.47 – -1.87	**<0.001**	PA no TA1	6	0.44	-1.01 – 2.00	0.520
FTA2	28	-2.25	-3.17 – -1.33	**<0.001**	PTA2	34	-1.86	-2.67 – -1.05	**<0.001**	PA no TA2	6	-0.11	-1.62 – 1.39	0.881
FTA99	120	-1.99	-2.92 – -1.06	**<0.001**	PTA99	102	-0.63	-1.43 – 0.18	0.128					
Random effects					**Random effects**					**Random effects**				
Variance (σ^2)		2.30			Variance (σ^2)		2.24			Variance (σ^2)		1.77		
Foot : Sheep ID		0.73			Foot : Sheep ID		0.69			Foot : Sheep ID		2.19		
Sheep ID		0.00			Sheep ID		0.00			Sheep ID		0.00		
Week		0.97			Week		0.70			Week		0.00		
ICC		0.43			ICC		0.38			ICC		-		
No. Foot		4			No. Foot		4			No. Foot		3		
No. Sheep ID		20			No. Sheep ID		20			No. Sheep ID		3		
No. Week		34			No. Week		34			No. Week		4		
Observations		204			Observations		204			Observations		18		
Marginal R^2		0.124			Marginal R^2		0.186			Marginal R^2		0.040		
Conditional R^2		0.496			Conditional R^2		0.498			Conditional R^2		NA		

CI: Confidence intervals.

FTA: Foot trim plus topical antibiotic foot spray.

ICC: Intraclass correlation coefficient.

PTA: Parenteral antibiotics plus topical antibiotic foot spray.

4.2 Key virulence factors, *aprB2*, *aprV2* and *fimA* of *Dichelobacter nodosus* and associations with the severity of footrot: Bristol study samples

4.2.1 *Dichelobacter nodosus* serogroups detected in swab DNA from the subset of 25 ewes from the Bristol study flock and comparison with those found in isolate DNA from the Bristol study flock

Serogroup detection from swab DNA carried out in the current study was compared with serogroup detection by multiplex PCR from previous analysis of 268 fully typable isolates from 157 interdigital skin and 35 non-interdigital lesion samples from the Bristol study (Smith *et al.*, 2017). Of the 578 swab DNA samples positive for *D. nodosus*, 411 were analysed using singleplex serogroup PCR. There were 409 samples (393 interdigital skin samples from all 25 ewes and 16 non-interdigital lesion samples from 10 of the 25 ewes) which yielded detection of at least one serogroup.

The same four serogroups were detected in swab DNA as in the isolate analysis from Smith *et al.* (2017). These were B, D, H and I. In both swab DNA and isolate DNA from Smith *et al.* (2017) the most common serogroup detected was B followed by H then I and finally D (**Table 3.5**). However, the proportion of samples positive for serogroups H, I and D were significantly higher in swab DNA than isolate DNA analysis, particularly for serogroup H, Cochran-Mantel-Haenszel test (**Table 3.5**). In swab DNA it was most common to detect two serogroups in a sample, frequently B and H (**Table 3.6**). In isolate DNA serogroup B alone was most frequently detected (Smith *et al.*, 2017).

Table 4.18: Number and percentage of samples positive for each serogroup detected from swab DNA analysis (from the current study) and isolate DNA analysis (from Smith *et al.*, 2017) with Cochran-Mantel-Haenszel test calculated for each serogroup separately.

Source	No. of samples positive (%)	No. of samples negative	Total	Odds ratio	CMH statistic	DoF	p value
Serogroup B							
DNA	346 (84.6)	63	409				
Isolate	226 (84.3)	42	268				
Total	572	105	677	1.02	< 0.01	1	0.99
Serogroup H							
DNA	331 (80.9)	78	409				
Isolate	23 (8.6)	245	268				
Total	354	323	677	44.07	333.19	1	**< 0.0001**
Serogroup I							
DNA	63 (15.4)	346	409				
Isolate	16 (6.0)	252	268				
Total	79	598	677	2.87	13.06	1	**0.0003**
Serogroup D							
DNA	21 (5.1)	388	409				
Isolate	3 (1.1)	265	268				
Total	24	653	677	4.78	6.49	1	**0.0108**

Table 4.19: Number and percentage of swab DNA samples by each combination of serogroups detected.

Serogroups detected	**1**	**2**	**3**	**4**	
No.	107	255	44	3	
Serogroup combinations	**No.**				**%**
B	70				65.4
H	33				30.8
I	2				1.9
D	2				1.9
BH		226			88.6
HI		25			9.8
BI		3			1.2
BD		1			0.4
BHI			29		65.9
BHD			14		31.8
HDI			1		2.3
BHID				3	100
%	**26.2**	**62.3**	**10.8**	**0.7**	

There was a significant difference in the mean ranks between the serogroups, p = 0.001 Kruskal-Wallis test (**Table 3.7**). The difference is indicated to be by serogroups I and D which show higher mean ranks than serogroups B and H (**Figure 3.1**).

Table 4.20: Comparison of median log_{10}(*D. nodosus* load +1) by serogroup detection using the Kruskal-Wallis test.

Serogroup	No. of samples	Median	Mean rank	H value	DoF	p value
B	346	4.59	369.0			
H	331	4.58	371.9			
I	63	4.90	451.2			
D	21	5.02	512.6			
				15.56	3	0.001

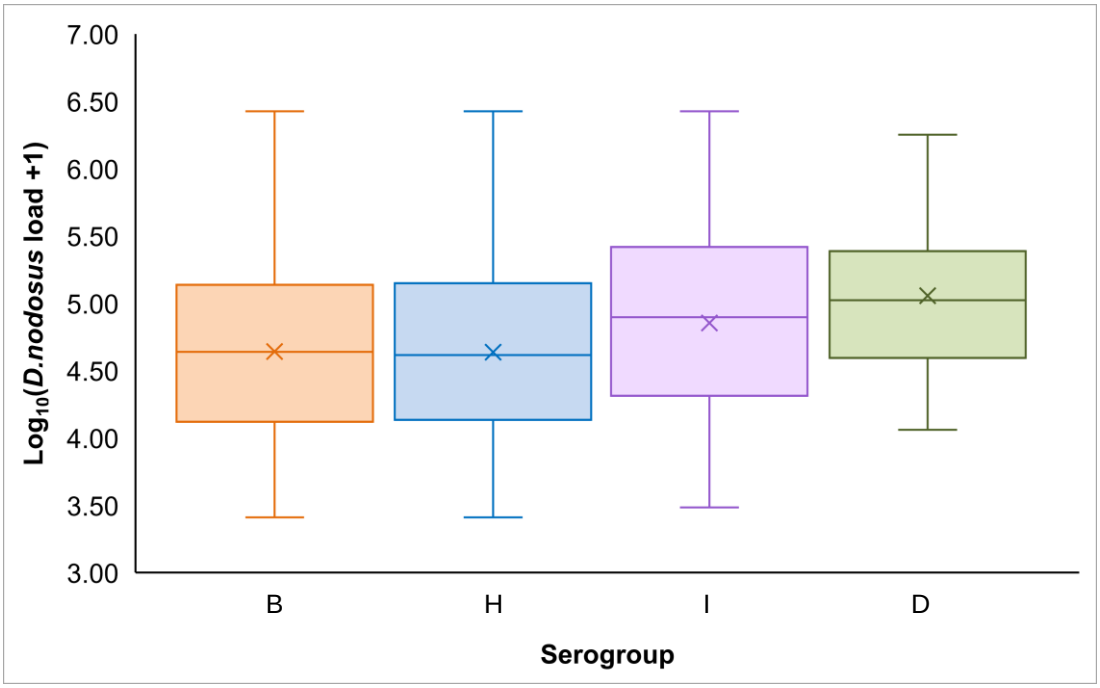


Figure 4.6: Box and whisker plot of median, mean (x), quartiles, and range for log_{10}(*D. nodosus* load +1) by serogroup.

There were 31 samples (all from the interdigital skin) where one or more *D. nodosus* serogroups were detected in DNA from cultured isolate(s) and from uncultured swab DNA of the same sample. The same serogroup(s) were detected in isolate and swab DNA in seven (23%) samples. For one sample (3%) serogroup B was detected in both isolate and swab DNA but serogroup I was also detected by isolate DNA and serogroup H was also detected by swab DNA. For 22 (71%) samples the same serogroup was detected from isolate DNA and swab DNA analysis but swab DNA analysis resulted in additional serogroups being detected. Finally, isolate DNA analysis and swab DNA analysis detected different serogroup(s) in one (3%) sample. Serogroup H was the additional serogroup in the majority of the 22 (17, 77%) samples where additional serogroups were detected in swab DNA compared to isolate DNA. Serogroups H and I were the additional serogroups detected

in three (14%) samples where additional serogroups were detected in swab DNA compared to isolate DNA. In the remaining two samples; in one (5%) sample serogroup D was the additional serogroup detected and in the other (5%) serogroup D and H were the additional serogroups detected in swab DNA compared to isolate DNA. From swab DNA analysis, there were 16 non-interdigital lesion samples from footrot lesions from 10 of the subset of 25 ewes. All 16 contained serogroup B and nine contained serogroup H. No other serogroups were detected.

4.2.1.1 Serogroup detection by interdigital dermatitis and severe footrot score in samples from the subset of 25 ewes

The highest percentage of swab DNA samples positive had ID score 4 for serogroups B and H, score 3 for I, and score 2 for D (**Table 3.8**). The were no significant associations between disease state and serogroup detection, p values > 0.05 BM (**Table 3.9** (binary disease state numbers) **Table 3.10** (ID score BM) & **3.11** (SFR score BM). Univariable results are given in **Appendix 5** (ID score) and **Appendix 6** (SFR score).

Table 4.21: Number and percentage of samples positive for each serogroup detected by ID score and SFR score.

Disease severity score	No. of samples	NO. OF SAMPLES (%) POSITIVE BY SEROGROUP							
		B		**H**		**I**		**D**	
Interdigital dermatitis									
0	209	172	(82.3)	160	(76.6)	32	(15.3)	8	(3.8)
1	85	68	(80)	75	(88.2)	11	(12.9)	5	(5.9)
2	47	42	(89.4)	37	(78.7)	8	(17.0)	4	(8.5)
3	29	27	(93.1)	23	(79.3)	9	(31.0)	2	(6.9)
4	39	37	(94.9)	36	(92.3)	3	(7.7)	2	(5.1)
Total	409	346		331		63		21	
Severe footrot									
0	316	264	(83.5)	261	(82.6)	50	(15.8)	19	(6.0)
1	93	82	(88.2)	70	(75.3)	13	(14.0)	1	(1.1)
Total	409	346		331		63		21	

Table 4.22: Number and percentage of samples positive for each serogroup detected by binary ID score and SFR score.

Disease severity score	No. of samples	NO. OF SAMPLES (%) POSITIVE BY SEROGROUP			
		B	**H**	**I**	**D**
Interdigital dermatitis					
0 - 1	294	240 (81.6)	235 (79.9)	43 (14.6)	13 (4.4)
2 - 4	115	106 (92.2)	96 (83.5)	20 (17.4)	8 (7.0)
Total	409	346	331	63	21
Severe footrot					
0	316	264 (83.5)	261 (82.6)	50 (15.8)	19 (6.0)
1	93	82 (88.2)	70 (75.3)	13 (14.0)	1 (1.1)
Total	409	346	331	63	21

Table 4.23: Multivariable binomial mixed effects regression model of *D. nodosus* serogroup detection on ID score ≥ 2.

			ID SCORE 2 - 4			
Predictors	**No.**	**%**	**Odds R.**	**CI**	**CS**	**p value**
B positive	106	92.2	1.90	0.77 – 4.64	1.40	0.161
H positive	96	83.5	1.18	0.58 – 2.41	0.45	0.652
I positive	20	17.4	1.17	0.53 – 2.58	0.39	0.693
D positive	8	7.0	1.17	0.39 – 3.58	0.28	0.778
Log_{10}(*D. nodosus* load +1)	409	100	1.81	1.17 – 2.82	2.66	**0.008**
References:						
B negative						
H negative						
I negative						
D negative						

Random effects	
Variance (σ^2)	3.29
Foot region : (Foot : Sheep ID)	0.00
Foot : Sheep ID	0.00
Sheep ID	0.48
Week	0.86
ICC	0.29
No. Foot region	2
No. Foot	4
No. Sheep ID	25
No. Week	38
Observations	409
Marginal R^2	0.058
Conditional R^2	0.330

CI: Confidence intervals.
CS: Coefficient statistic.
ICC: Intraclass correlation coefficient.
Odds R.: Odds Ratios.
%: Percentage of samples with ID score 2 – 4 positive by serogroup.

Table 4.24: Multivariable binomial mixed effects regression model of *D. nodosus* serogroup detection on positive SFR score.

			SFR SCORE 1			
Predictors	**No.**	**%**	**Odds R.**	**CI**	**CS**	**p value**
B positive	82	88.2	1.13	0.43 – 3.01	0.25	0.803
H positive	70	75.3	0.71	0.34 – 1.50	-0.89	0.374
I positive	13	14.0	1.80	0.66 – 4.93	1.15	0.251
D positive	1	1.1	0.25	0.04 – 1.48	-1.53	0.126
Log_{10}(*D. nodosus* load +1)	409	100	1.15	1.70 – 1.90	0.55	0.582
References:						
B negative						
H negative						
I negative						
D negative						

Random effects	
Variance (σ^2)	3.29
Foot region : (Foot : Sheep ID)	0.51
Foot : Sheep ID	0.81
Sheep ID	0.00
Week	0.76
ICC	0.39
No. Foot region	2
No. Foot	4
No. Sheep ID	25
No. Week	38
Observations	409
Marginal R^2	0.027
Conditional R^2	0.404

CI: Confidence intervals.
CS: Coefficient statistic.
ICC: Intraclass correlation coefficient.
Odds R.: Odds Ratios.
%: Percentage of samples with ID score 2 – 4 positive by serogroup.

4.2.2 Persistence of serogroups on feet over time from swab DNA analysis

The duration of detection of a serogroup on a foot with serogroup records for 13 of the routine sampling weeks (weeks 1-4, 13-16, 30-33, and 43) were investigated. Serogroup detection on 16 feet of 13 of the 25 ewes were analysed. Serogroup detection was ranked from 0; never detected, 1; detected in at least 1 and up to 10 routine sampling weeks, 2; detected in ≥ 11 of the routine sampling weeks, to 3; detected in all 13 routine sampling weeks. There was a significant difference in median number of feet by detection frequency between the four serogroups, $p < 0.01$ Kruskal-Wallis test (**Table 3.13**). Serogroups B and H appeared to persist but were also the most commonly detected. There was not enough data to investigate whether serogroup detection changed after treatment.

Table 4.25: Number of feet with each level of serogroup detection frequency by serogroup.

		NO. OF FEET BY SEROGROUP			
Detection frequency		**B**	**H**	**I**	**D**
0	Never detected	0	0	7	10
1	Detected in at least 1 and up to 10 routine sampling weeks	2	4	7	6
2	Detected in ≥ 11 of the routine sampling weeks	7	9	2	0
3	Detected in all 13 routine sampling weeks	7	3	0	0

4.2.3 Presence of *aprB2* and *aprV2* in clinically healthy foot samples

There were 124 healthy foot samples positive for *D. nodosus* tested for $\log_{10}$(*aprB2* and *aprV2* load). All but one was *aprV2* positive and two of these *aprV2* positive samples were also positive for *aprB2*. No samples were positive for *aprB2* alone. In the two samples that detected positive for both *aprV2* and *aprB2*, the *aprV2* load was higher; $\log_{10}$(*aprV2* load) was 5.07 and 6.00 and the $\log_{10}$(*aprB2* load) was 3.53 and 3.65 respectively for the two samples. These two samples were from different sheep and sampled only six days apart.

4.3 Key virulence factors and phylogenetic diversity of *Dichelobacter nodosus* and associations with the severity of footrot: On a single UK farm

4.3.1 Genomic analysis of *Dichelobacter nodosus* isolates from the Bristol study

A total of 303 *D. nodosus* isolates from the Bristol study passed sequencing quality control. Of these, a further 71 were removed as they were regarded as "duplicates". The number of ewes and lambs by disease severity scores for the 232 *D. nodosus* isolates is given in **Table 4.3**. Phylogenetic SNP analysis of the 232 Bristol study isolates revealed that all isolates fitted into three distinct clades which will be referred to as 1, 2, and 3 as highlighted in **Figures 4.2** and **4.3**.

Table 4.26: Number of *D. nodosus* isolates by ID and SFR score for ewes and lambs for the 232 Bristol isolates analysed.

Foot ID score, SFR score	No. of ewes	No. of lambs
0, 0	72	5
1, 0	27	1
2, 0	18	1
3, 0	12	5
4, 0	28	9
0, 1	17	34
0, 2	0	2
Unknown	1	0
TOTAL	**175**	**57**

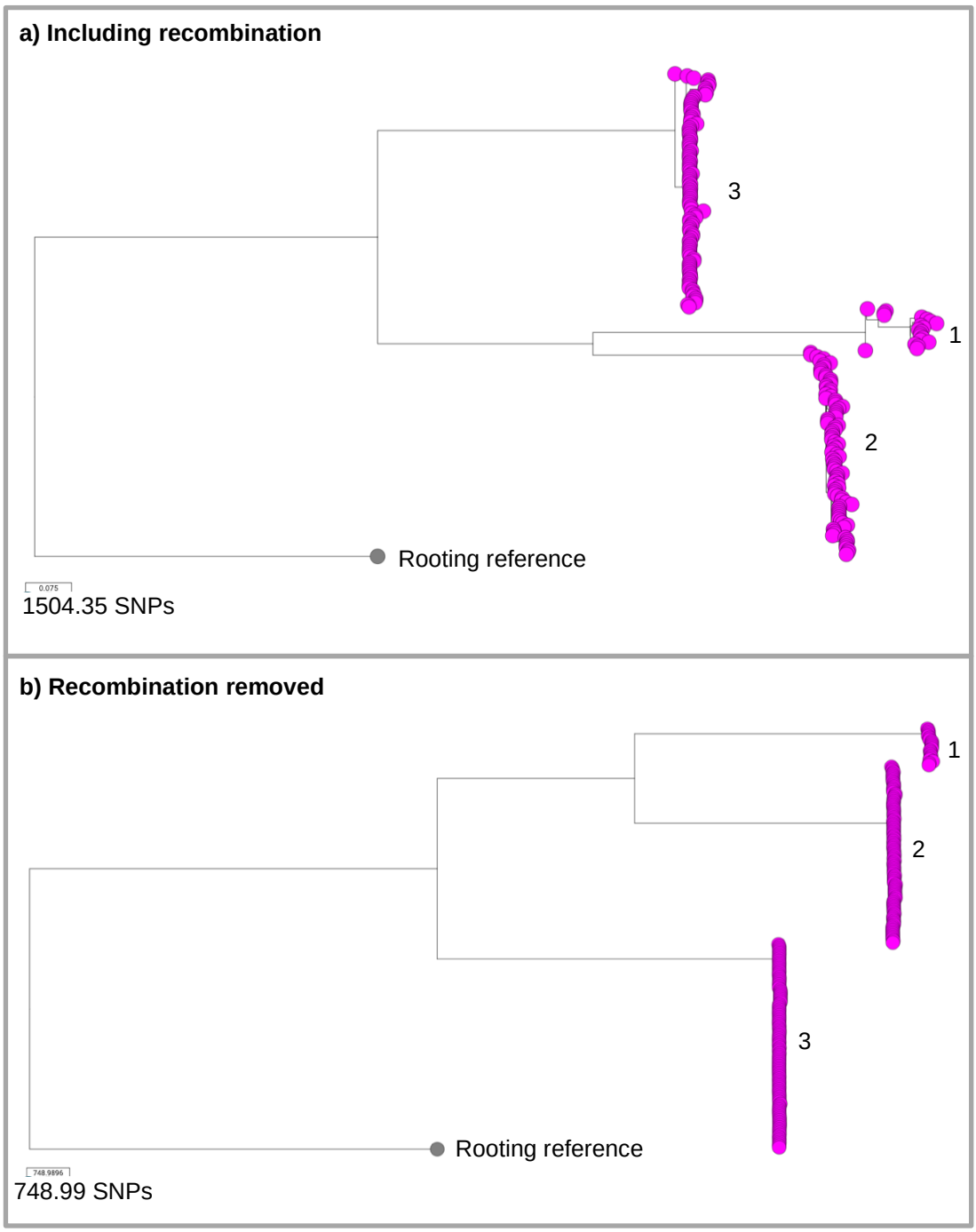


Figure 4.7: Maximum likelihood trees showing clades 1, 2 and 3 of the *D. nodosus* isolates (232) from the Bristol study, rooted using the *D. nodosus* VCS1703A reference including recombination (a) and with recombination removed (b).

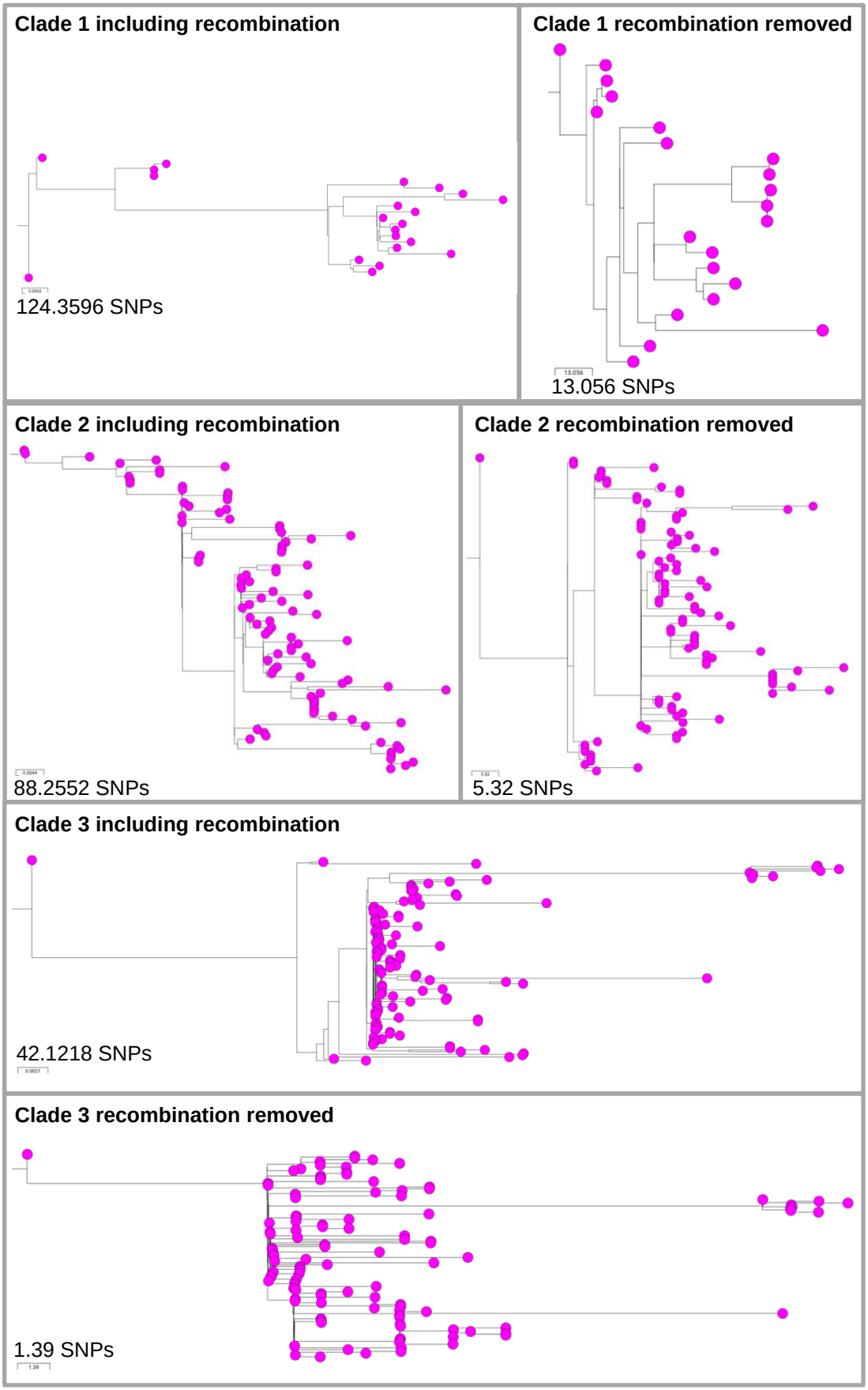


Figure 4.8: Closer view of clades 1, 2 and 3 from Figure 4.2.

4.3.2 Recombination

There was a considerable frequency of recombination events occurring within the three clades demonstrated in **Figure 4.4,** with the majority occurring across multiple isolates. Recombination rates (ρ/θ) and the probability of a site being altered by recombination or mutation (r/m) are given for each Bristol clade in **Table 4.4**. Peaks in recombination occurred for genes *fimA*, *fimB*, *omp1A*, plus an unannotated gene highlighted in **Figure 4.4**. There was an indication of different recombination events occurring between different serogroups identified within the three clades (**Figure 4.4**).

Table 4.27: Number of SNPs detected in the Bristol samples before and after recombination was removed.

Bristol samples	No. of samples	Total no. of SNPs	Total no. of vertically inherited SNPs	Total r/m	Mean r/m	Total ρ/θ	Mean ρ/θ
All	232	20058	7413 (37.0%)	2559.22	5.53	132.67	0.29
Clade 1	21	2987	252 (8.4%)	454.31	11.36	26.41	0.66
Clade 2	98	5037	403 (8.0%)	1115.96	5.75	60.67	0.31
Clade 3	113	3580	189 (5.3%)	1021.98	4.56	46.59	0.21

r/m: Number of homologous SNPs in homologous recombination regions per vertically inherited SNP (mutation).
ρ/θ: Number of homologous recombination events per vertically inherited SNP (mutation).

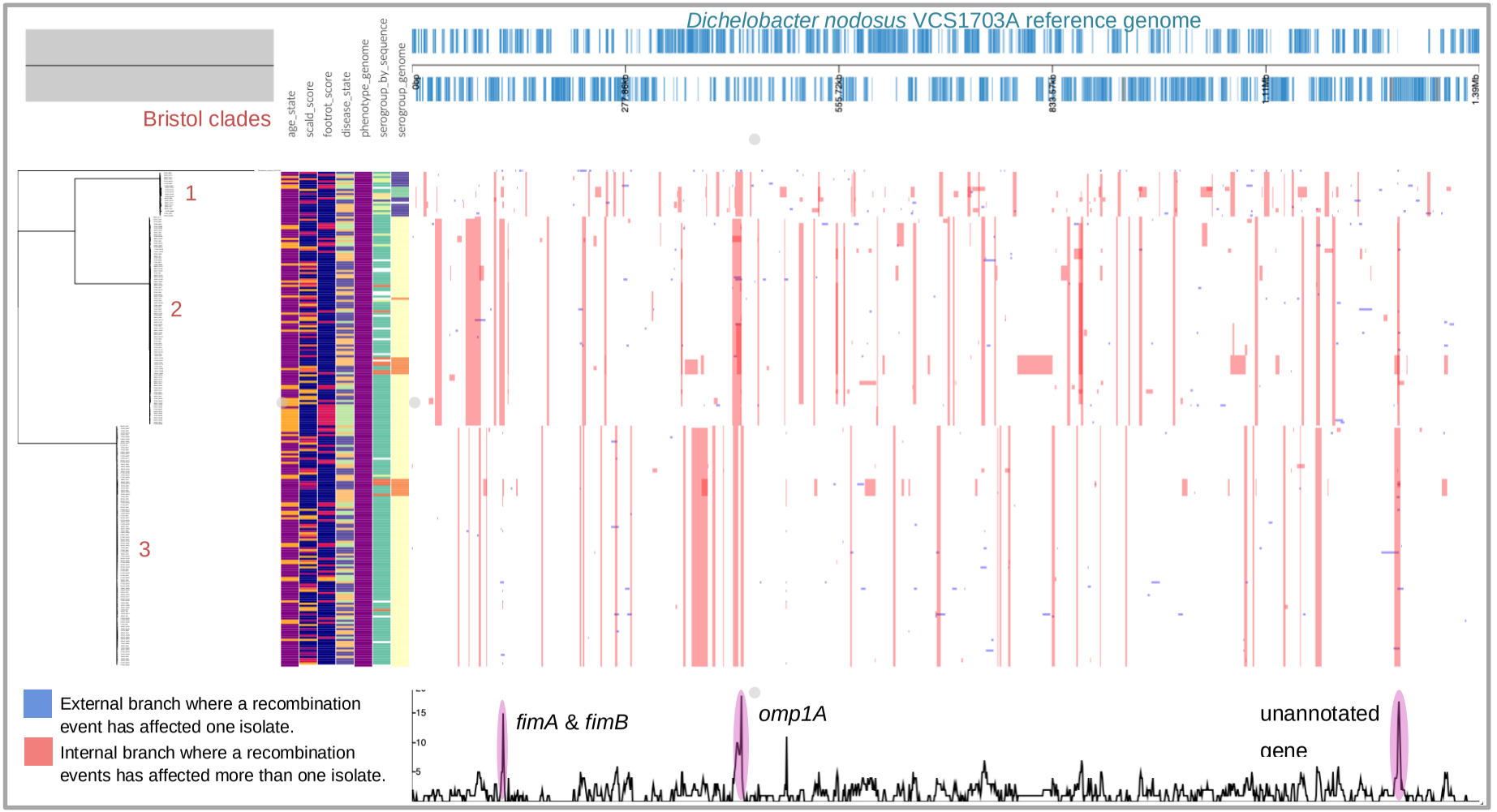


Figure 4.9: Recombination predictions along 1.39Mb from the maximum likelihood SNP tree of 232 Bristol *D. nodosus* isolates with peaks in recombination highlighted and identified through searching the reference genome.

4.3.3 Acidic protease identification from laboratory and genomic analysis

In all isolates analysed from the Bristol study, the highest percentage identity was with *aprV2*, therefore no further analysis was done regarding *aprV2*/*B2*.

4.3.4 Fimbrial serogroup identification from laboratory and genomic analysis

Serogroups identified through laboratory multiplex PCR (Smith *et al.*, 2017) and genomic analysis are summarised in **Table 4.5**. The same four serogroups; B, D, H and I were identified in both laboratory and genomic analysis. Serogroup identification through laboratory and genomic analysis matched in 192/232 (82.8%) Bristol flock *D. nodosus* isolates. One isolate was identified as both serogroup B and H through laboratory analysis and serogroup H through genomic analysis. Of these 192, percentage nucleotide similarity in 190 was > 95%. The three clades were visualised by serogroup identified by laboratory tests and genomic analysis for comparison, shown in **Figures 4.5**, **4.6** and **4.7**. Although very similar to laboratory serogroup analysis, the phylogenetic pattern of serogroup identification by genomic analysis indicated greater coherence. The most distal branch comprised *D. nodosus* isolates of a different serogroup in all three clades.

Genomic serogroup detection along with foot ID and SFR score and whether the sheep was a ewe or a lamb is shown in **Figures 4.8**, **4.9** and **4.10** for each clade. Any associations between serogroup and disease state or whether the sheep was a ewe or a lamb could not be identified as the data were too sparse to statistical model with such a complex random structure.

Table 4.28: Number of serogroups identified through laboratory and genomic analysis in each of the three clades of the 232 Bristol study *D. nodosus* isolates.

Serogroup	No. identified by lab analysis	No. identified by genomic analysis	No. where lab & genomic analysis matched
Clade 1			
B	9	0	0
D	2	5	2
H	8	15	7
B & H	1	0	0
Total	20	20	9
Unknown	1	1	-
Clade 2			
B	102	105	98
H	1	0	0
I	5	8	4
Total	108	113	102
Unknown	5	0	-
Clade 3			
B	78	89	76
H	3	0	0
I	7	9	5
Total	88	98	81
Unknown	10	0	-
Total identified	216	231	192

Figure 4.10: Bristol study clade 1 *D. nodosus* isolates by serogroup identified through laboratory (a) and genomic analysis (b). Tree with recombination removed and rooted using two *D. nodosus* isolates not from the Bristol study coloured by serogroup identified.

Orange: B, *lime green*: D, *purple*: H, *brown*: both B and H, *grey*: no serogroup identified or not analysed.

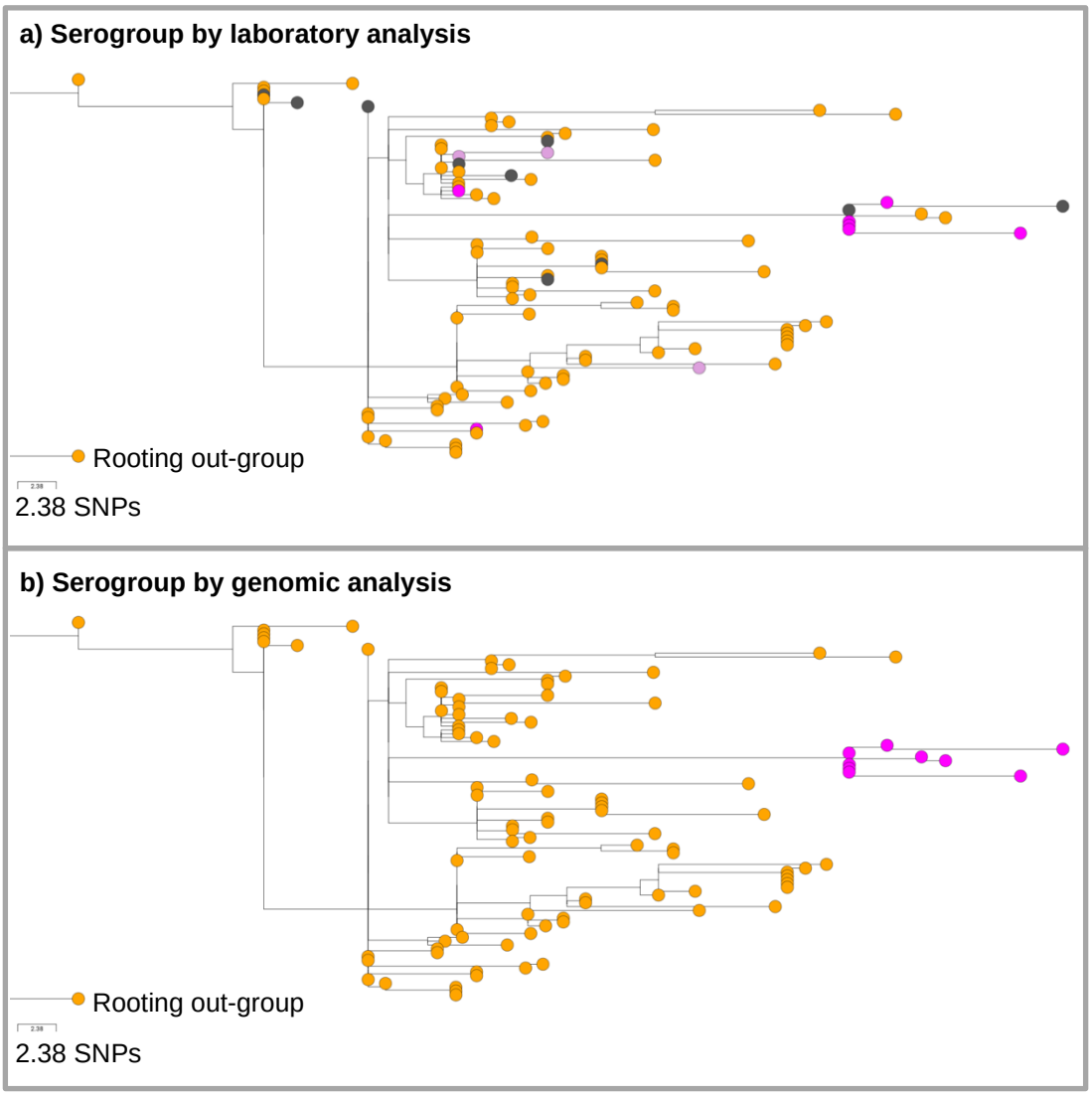


Figure 4.11: Bristol study clade 2 *D. nodosus* isolates by serogroup identified through laboratory (a) and genomic analysis (b). Tree with recombination removed and rooted using a *D. nodosus* isolate not from the Bristol study coloured by serogroup identified.

Orange: B, *purple*: H, *magenta*: I, *grey*: no serogroup identified or not analysed.

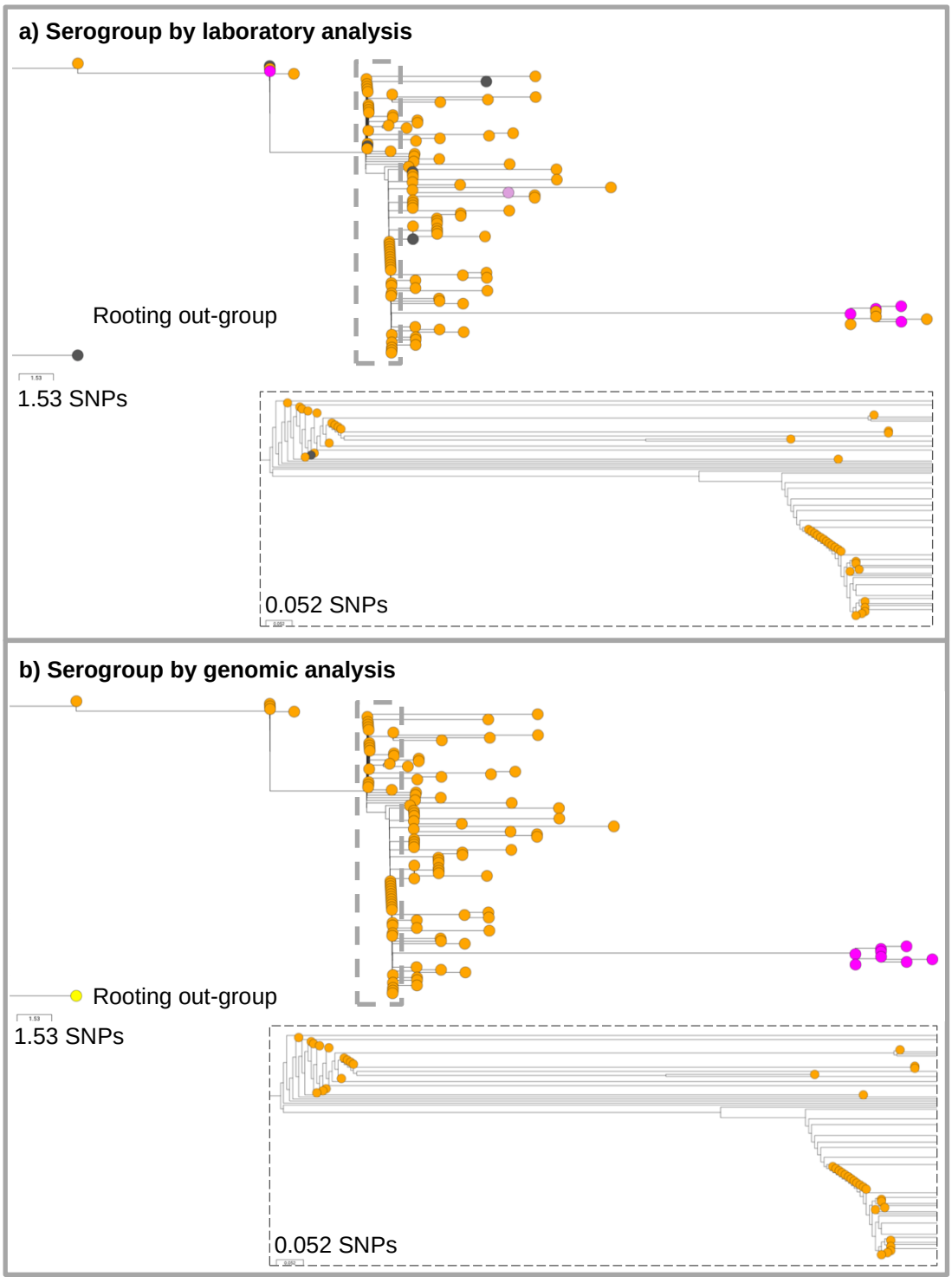


Figure 4.12: Bristol study clade 3 *D. nodosus* isolates by serogroup identified through laboratory (a) and genomic analysis (b). Tree with recombination removed and rooted using a *D. nodosus* isolate not from the Bristol study coloured by serogroup identified.

Orange: B, *yellow*: C, *purple*: H, *magenta*: I, *grey*: no serogroup identified or not analysed, *grey dash*: a closer view of the centre branch diversity.

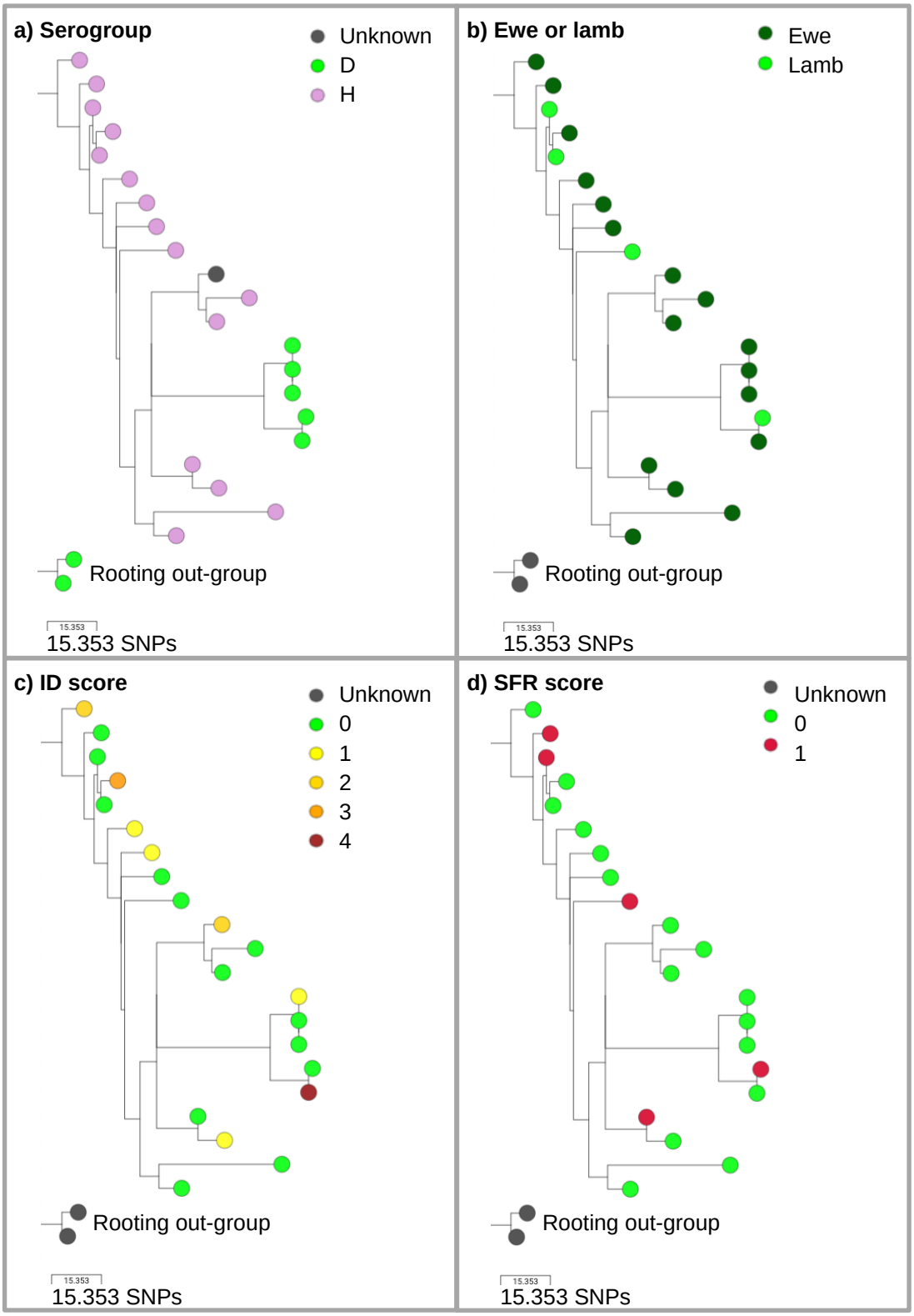


Figure 4.13: Bristol study clade 1 *D. nodosus* isolates by serogroup identified from genomic analysis (a), by sheep age category (b), by ID score (c) and by SFR score (d). Trees with recombination removed and rooted using a *D. nodosus* isolate not from the Bristol study.

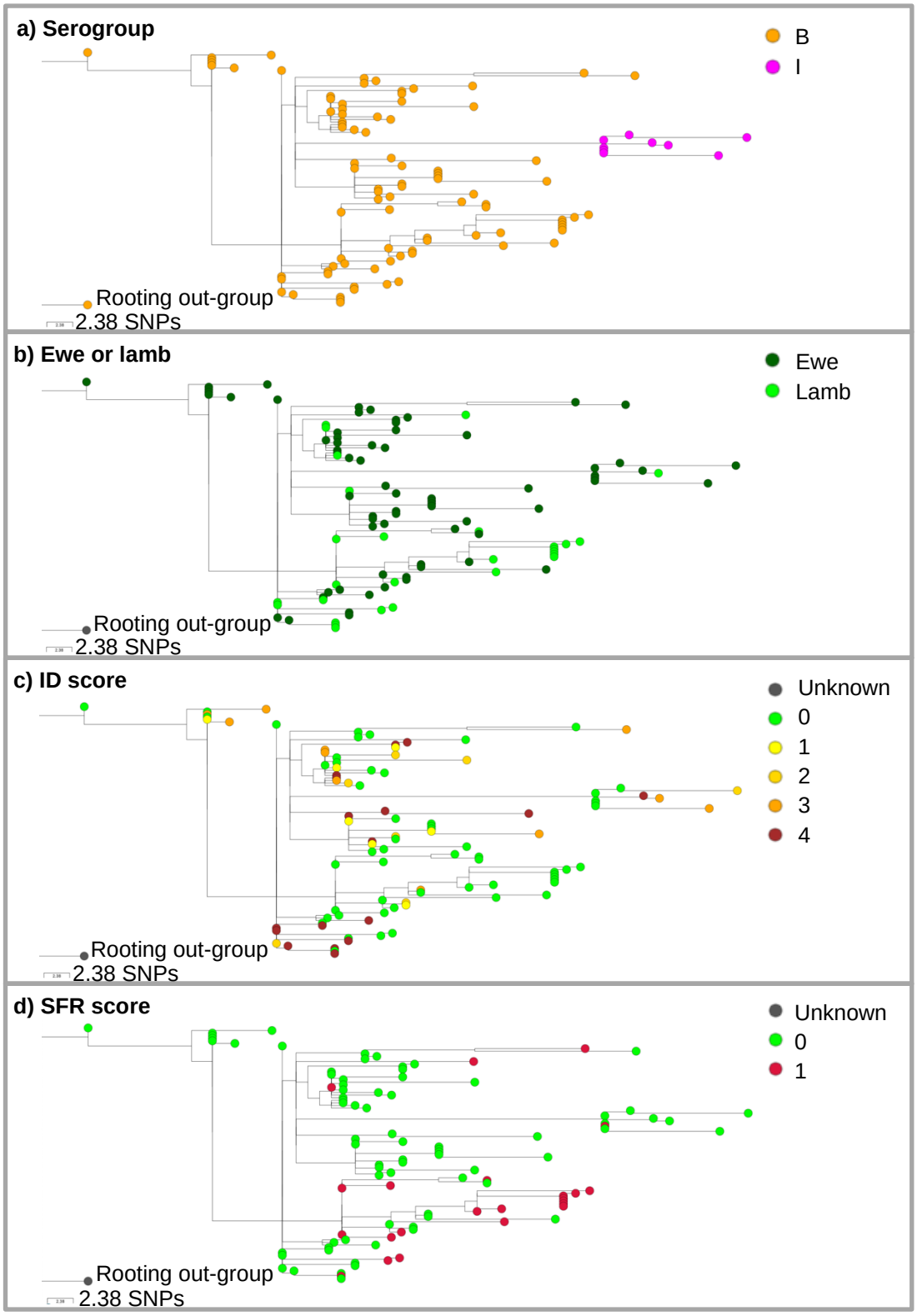


Figure 4.14: Bristol study clade 2 *D. nodosus* isolates by serogroup identified from genomic analysis (a), by sheep age category (b), by ID score (c) and by SFR score (d). Tree with recombination removed and rooted using a *D. nodosus* isolate not from the Bristol study.

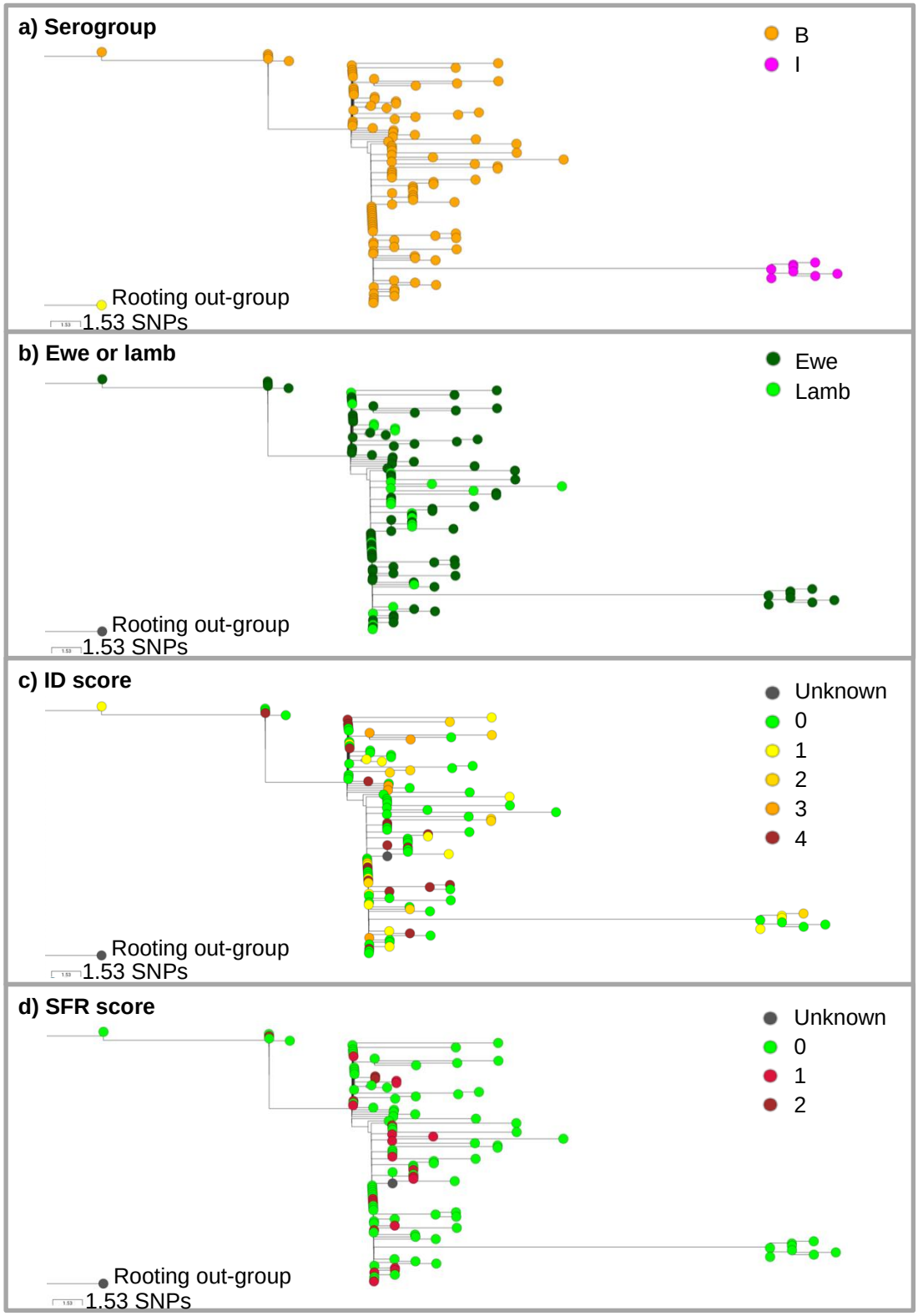


Figure 4.15: Bristol study clade 3 *D. nodosus* isolates by serogroup identified from genomic analysis (a), by sheep age category (b), by ID score (c) and by SFR score (d). Tree with recombination removed and rooted using a *D. nodosus* isolate not from the Bristol study.

4.3.5 Temporal signal analysis

Results from the temporal signal root-to-tip analysis of the Bristol flock clades are shown in **Table 4.6**, **Figures 4.11**, **4.12**, and **4.13**. An indication of temporal signal was shown for clade 1 from *TempEst* 'best fitting root'.

Table 4.29: Correlation coefficients (CC) and R^2 values from *TempEst* temporal root- to-tip analysis for each clade.

	OUT-GROUP ROOTED		*TEMPEST* BEST FITTING ROOT	
Clade	**CC**	**R^2**	**CC**	**R^2**
1	0.18	3.0757E-2	**0.61**	**0.37**
2	-0.09	7.7859E-3	0.20	3.9232E-2
3	0.30	8.819E-2	0.37	0.14

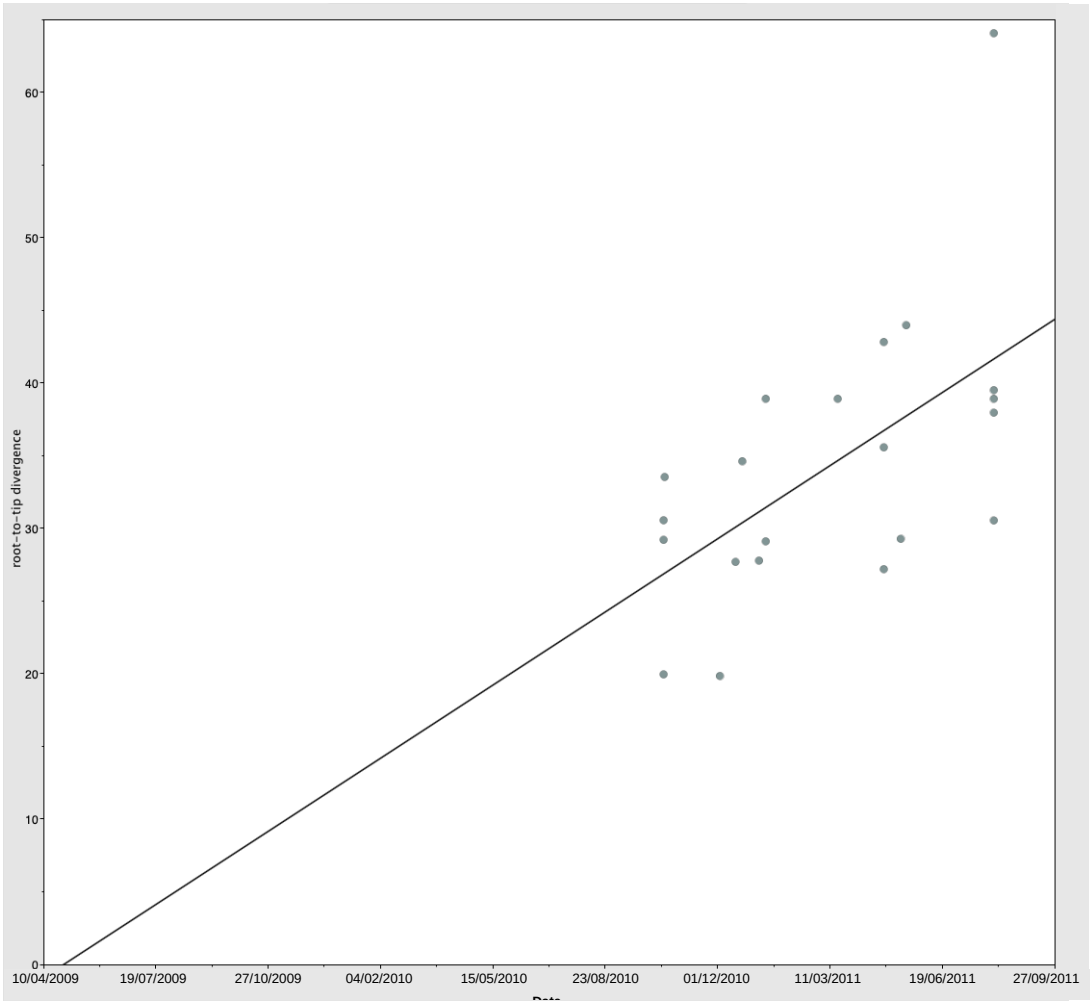


Figure 4.16: Root-to-tip linear regression of the genetic distances between *D. nodosus* isolates in Bristol farm clade 1 by date excluding rooting outgroup.

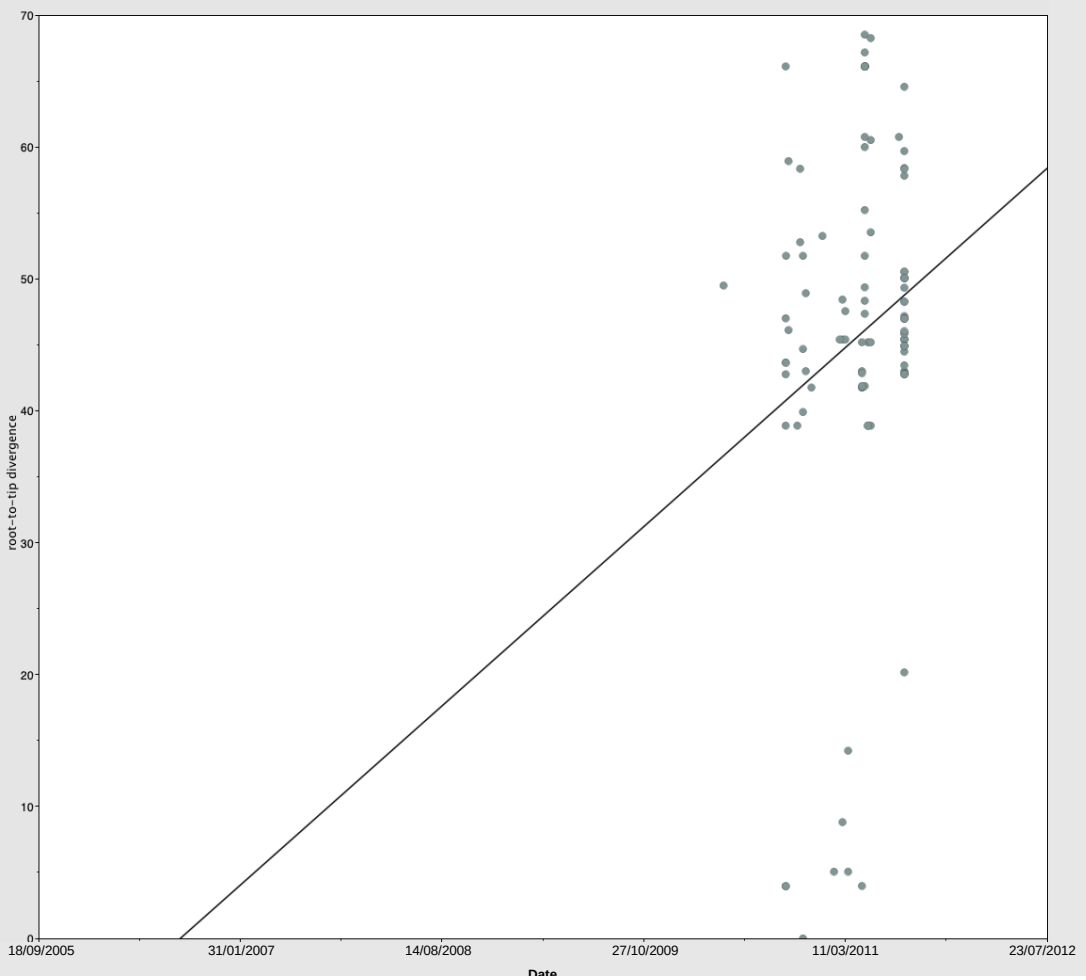


Figure 4.17: Root-to-tip linear regression of the genetic distances between *D. nodosus* isolates in Bristol farm clade 2 by date excluding rooting outgroup.

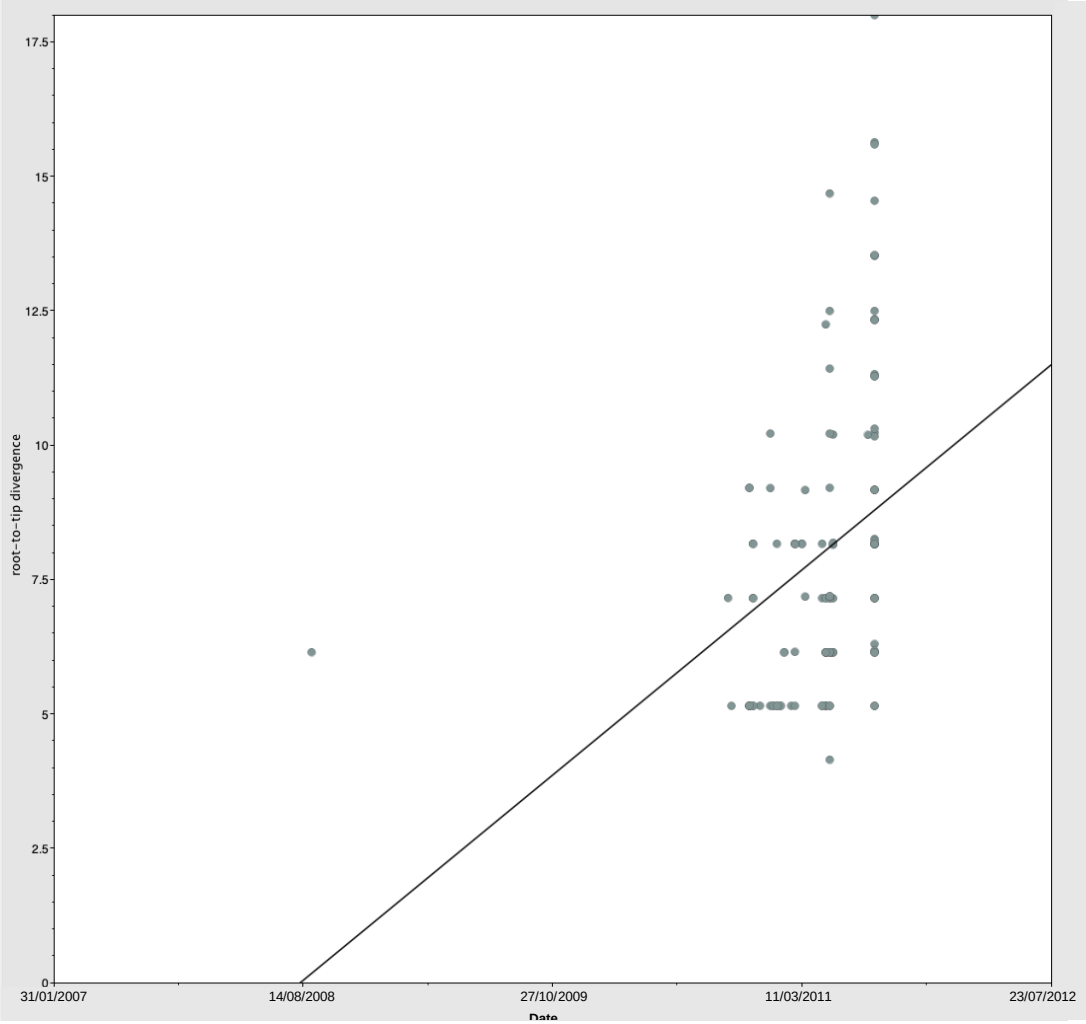


Figure 4.18: Root-to-tip linear regression of the genetic distances between *D. nodosus* isolates in Bristol farm clade 3 by date excluding rooting outgroup.

A significant temporal signal was identified in clade 1 using the *TempEst* 'best fitting root' tree where recombination was removed (**Figure 4.14**), p = 6.00e-04, R^2 = 0.47, lower DIC of 201.62 compared to a DIC of 247.83 when sampling dates were forced to be equal, confidence intervals shown in **Figure 4.15**. A rate of 14 substitutions/mutations occurring each year was identified (**Figure 4.14**). The most recent common ancestor was predicted to have emerged in 1998. MCMC parameter traces are shown in **Figure 4.16**.

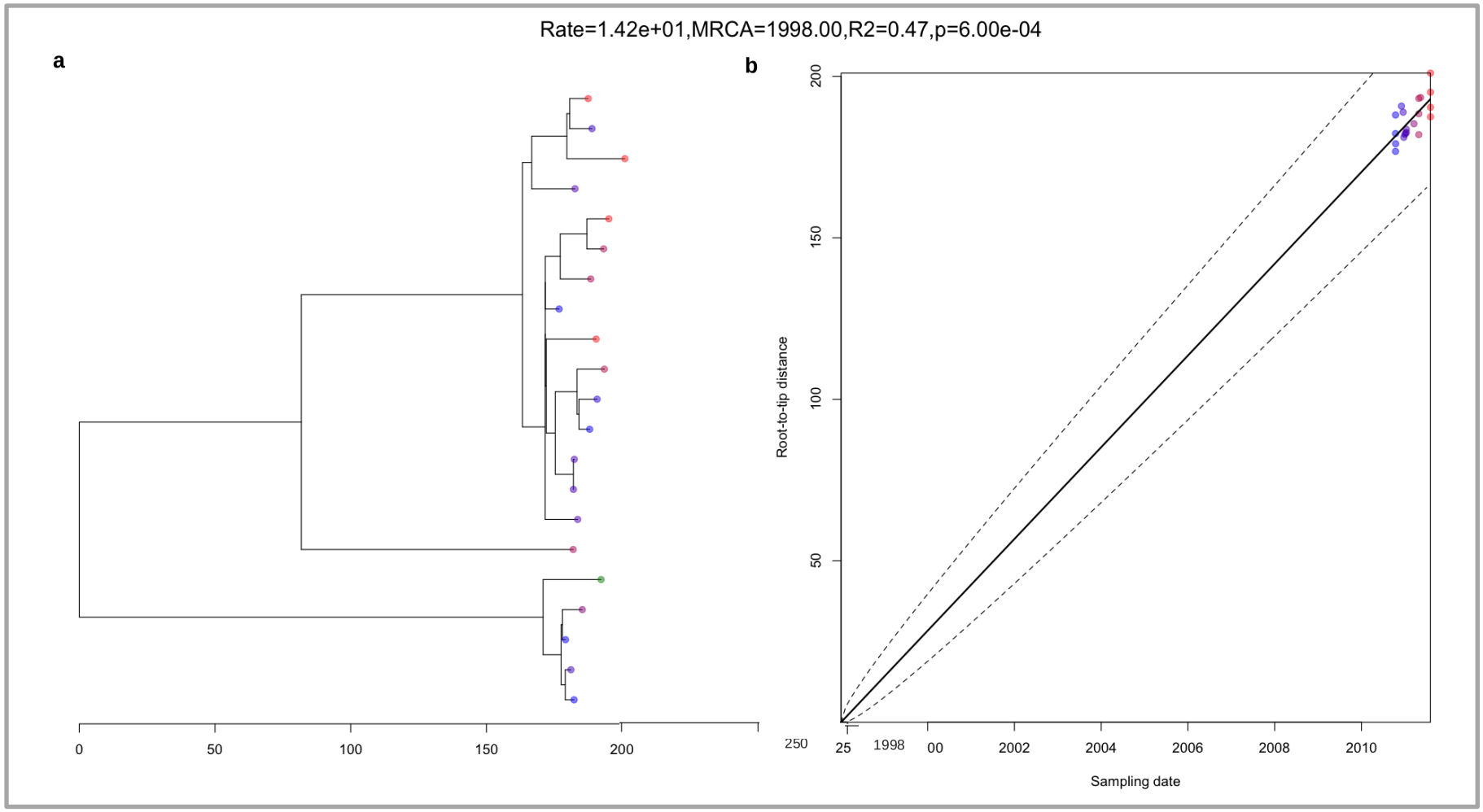


Figure 4.19: Bayesian root to tip analysis of Bristol clade 1, excluding the rooting outgroup, indicating the number of substitutions/mutations away from the root (a) and the date of the most recent common ancestor (MRCA) (b).

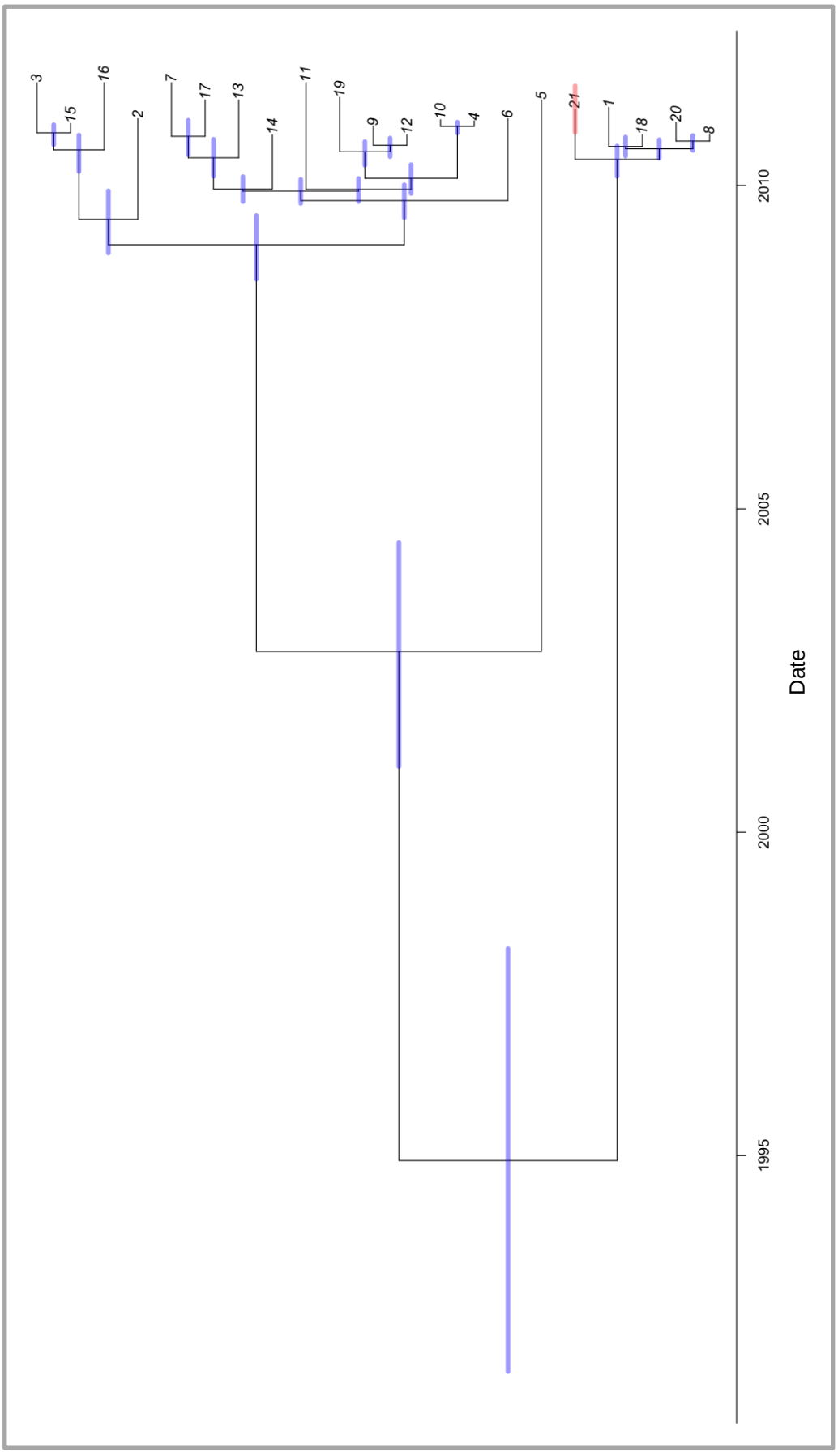


Figure 4.20: Confidence intervals of Bristol clade 1 temporal analysis.

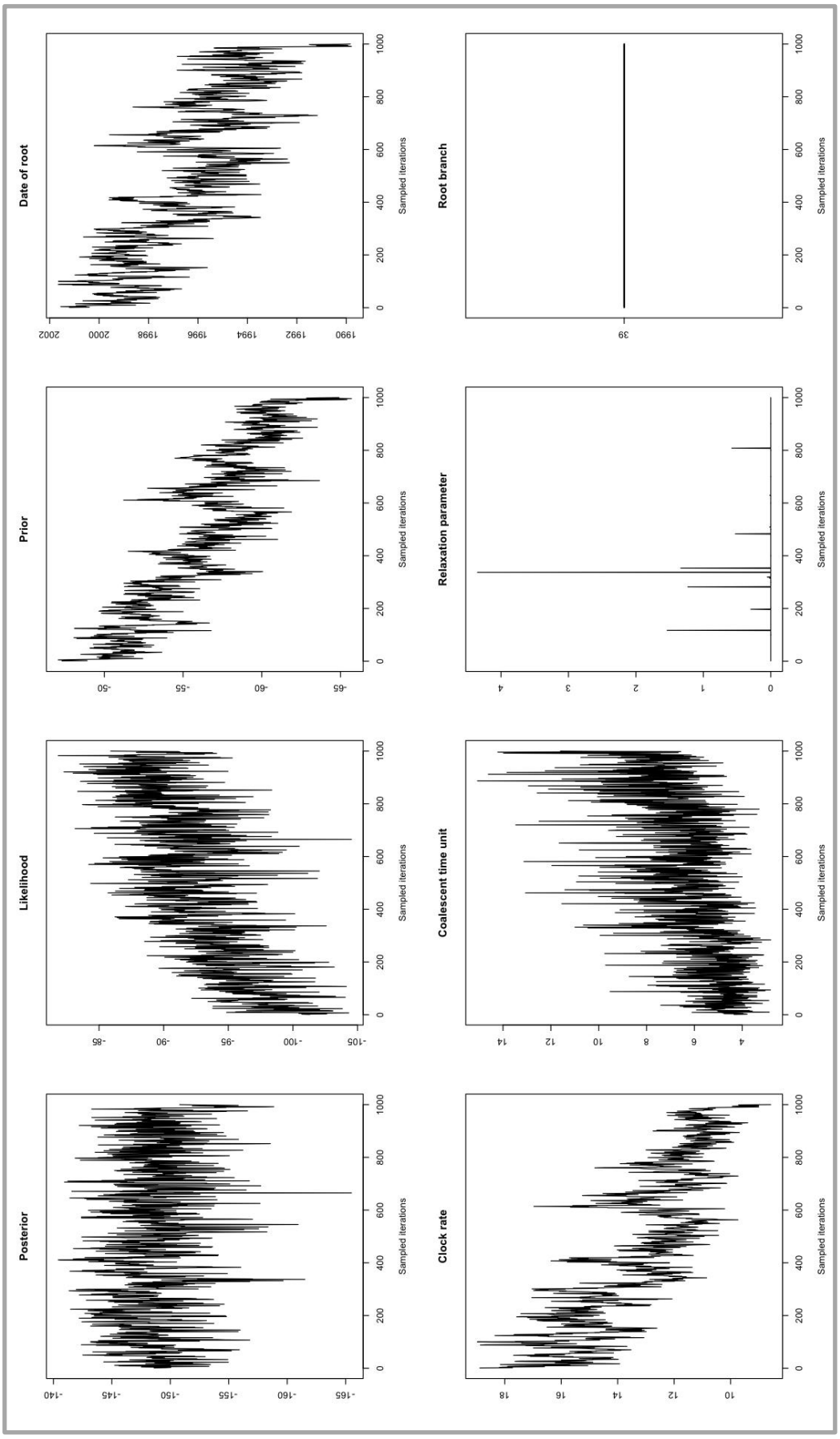


Figure 4.21: Bristol clade 1 MCMC parameter traces from simulating the phylogenetic tree of the number of substitutions.

4.4 Key virulence factors and phylogenetic diversity of *Dichelobacter nodosus* and associations with the severity of footrot: Globally sourced isolates

4.4.1 Genomic analysis of globally sourced *Dichelobacter nodosus* isolates

A total of 481 *D. nodosus* isolates were submitted for whole genome sequencing. These plus the 180 *D. nodosus* isolates already sequenced from Kennan *et al.* (2014) and Blanchard *et al.* (2018) and put through quality control. Of these 661, 599 matched to *Dichelobacter nodosus VCS1703A* (**accession no. CP000513**) and underwent quality control where 567 passed quality control with numbers from each country and animal species given in **Table 5.1**.

Table 4.30: Country and species origin of *D. nodosus* isolates that passed sequence quality control (Smith *et al.*, 2017, Blanchard *et al.*, 2018, Kennan *et al.*, 2014).

Country	No. of samples	No. from sheep	No. from cows	No. from goats
UK	397*	397*	0	0
Norway	52	45	6	1
Australia	40	40	0	0
Sweden	34	28	4	2
Germany	33	33	0	0
Denmark	8	8	0	0
Bhutan	1	1	0	0
India	1	1	0	0
Nepal	1	1	0	0

*303/397 (76%) are from the Bristol farm, the focus of this thesis (Smith *et al.*, 2017).

Key statistics from the 567 *D. nodosus* sequences included a median total sequence length of 1316417bp and median genome coverage of 93.65%. Depth of coverage (minimum fragment length for assembly) ranged from 25.22x – 1245.3x with a median of 218.67x. The number of contigs in 551/567 *D. nodosus* sequences ranged from 5 – 99 with a median of 16. Although no limit was set on the number of contigs in a sequence during quality control, there were 16 *D. nodosus* sequences with > 100 contigs; 105, 107, 112, 167, 175, 184, 230, 245, 390, 559, 608, 671, 695, 1059, 1220, 1287. There was also a median of 66 heterozygous SNPs. A total of 76/567 (71 from the Bristol study flock) *D. nodosus* isolates were duplicates and removed from analyses leaving 491 *D. nodosus* genomes.

4.4.2 SNP analysis of *Dichelobacter nodosus* genomes

Phylogenetic representation of the 491 globally sourced *D. nodosus* isolates with predicted recombination regions removed is given in **Figure 5.1**.

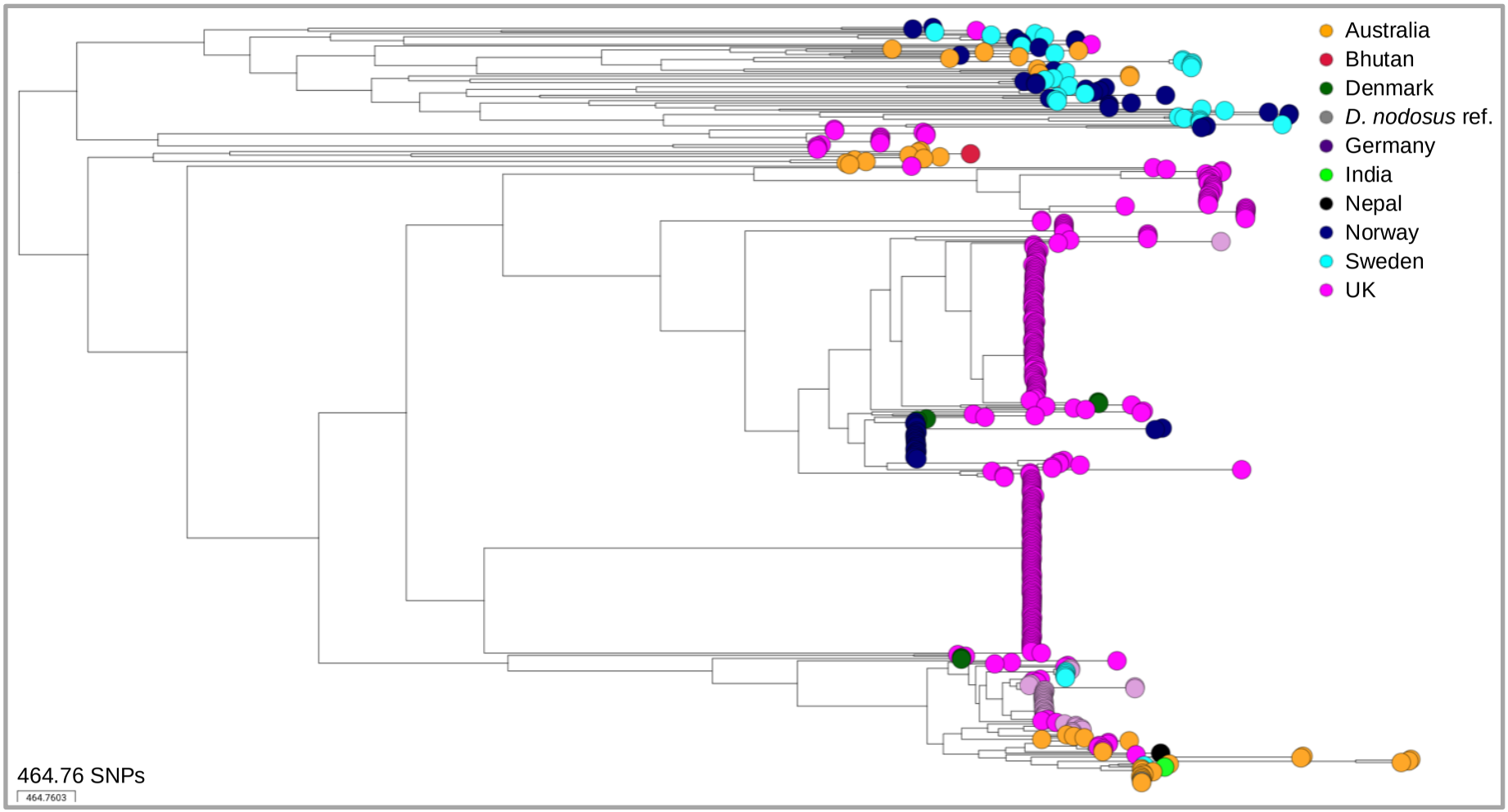


Figure 4.22: Maximum likelihood SNP tree of 491 *D. nodosus* isolates plus the mapping reference *D. nodosus* VCS1703A with recombination removed and rooted using *TempEst* 'best fitting root', isolates coloured by country of origin.

A large number of the UK isolates are from the Bristol flock study. As this is only one farm, a smaller selection of isolates from this farm were selected after genomic serogroup analysis. The 232 Bristol flock isolates formed three clades; 21 in clade 1, 98 in clade 2 and 113 in clade 3. From genomic analysis all were *aprV2* positive and the proportion of serogroups detected are shown in **Chapter 4, Table 4.4**. From this serogroup analysis 16 *D. nodosus* isolates from the Bristol flock study were selected to represent *D. nodosus* on the Bristol farm. Two serogroup D isolates, one from a healthy foot and one from a foot with a form of footrot, and two serogroup H isolates, one from a healthy foot and one from a foot with a form of footrot were selected from clade 1. Four serogroup B isolates, two from a healthy foot and two from a foot with a form of footrot, and two serogroup I isolates, one from a healthy foot and one from a foot with a form of footrot were selected from clade 2. The same was also selected from clade 3 as clade 2.

This resulted in 275 D. nodosus isolates from nine countries (**Figure 5.2**) *which were investigated further.*

The 13 *D. nodosus* isolates sampled from a cow or goat from Sweden and Norway are spread amongst the *D. nodosus* isolates sampled from sheep from Sweden and Norway showing no clear distinction in species sampled (**Figure 5.3**). The numbers of isolates from a cow or goat are too small to investigate further.

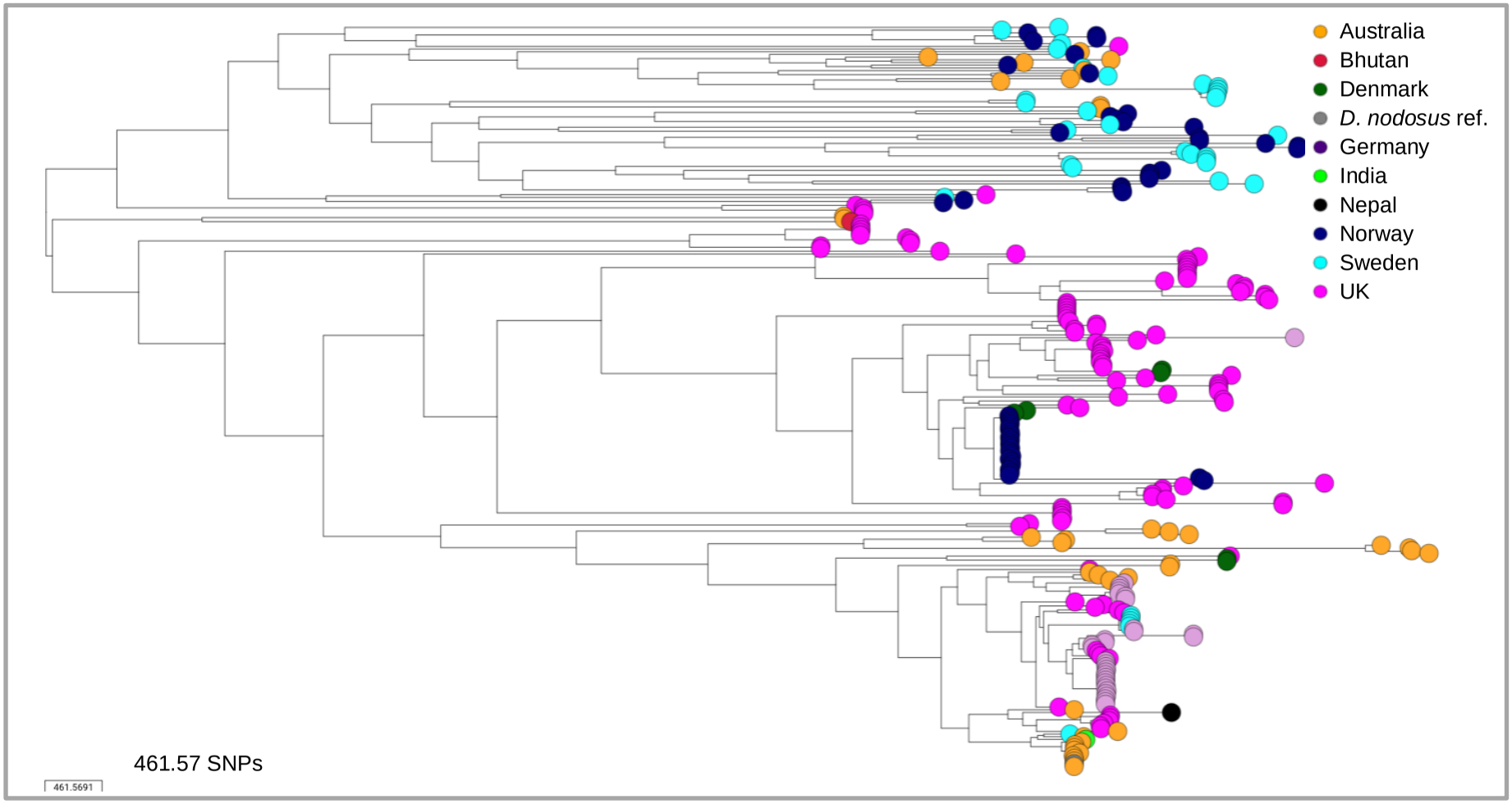


Figure 4.23: Maximum likelihood SNP tree of 275 *D. nodosus* isolates plus the mapping reference *D. nodosus* VCS1703A with recombination removed and rooted using *TempEst* 'best fitting root', isolates coloured by country of origin.

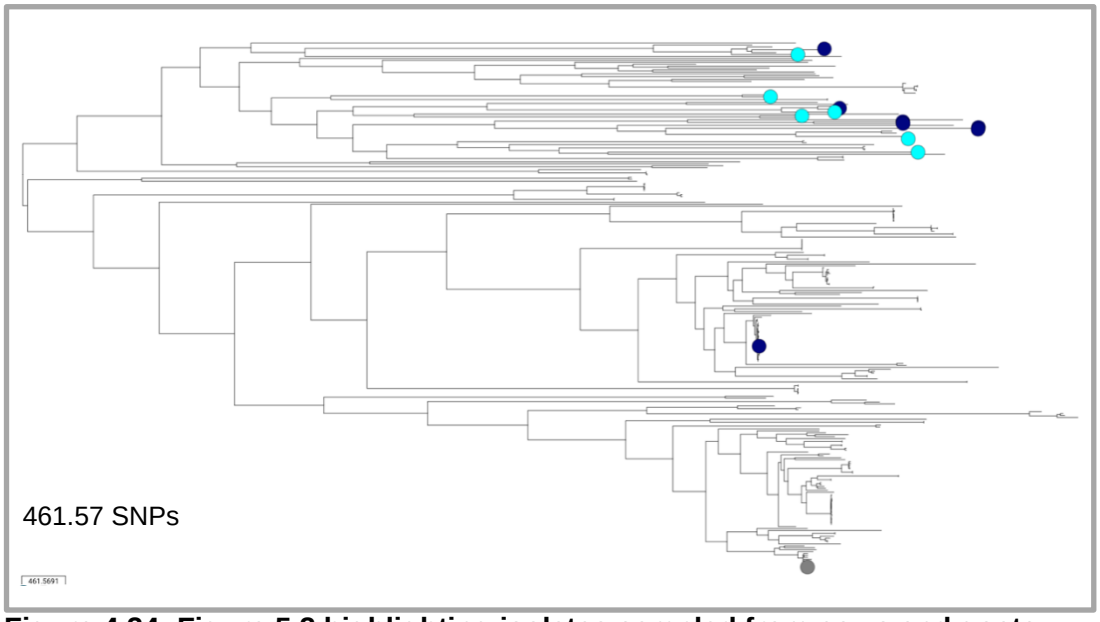


Figure 4.24: Figure 5.2 highlighting isolates sampled from cows and goats.

Turquoise: Sweden, *navy*: Norway, *grey*: *D. nodosus* VCS1703A reference.

4.4.3 Acidic protease identification

This section used the group of 275 global *D. nodosus* isolates with representative isolates from the Bristol farm. Laboratory analysis for acidic protease presence was available for 159/275 globally sourced *D. nodosus* isolates. Of these 159, 109 were positive for *aprV2* and 50 were positive for *aprB2*. Genomic analysis yielded acidic protease identification in 274/275 *D. nodosus* isolates, 68 *aprB2* positive and 206 *aprV2* positive (**Figure 5.4**). All 109 *D. nodosus* isolates positive for *aprV2* by laboratory analysis were also *aprV2* positive by genomic analysis. All but one (positive for *aprV2*) of the 50 *D. nodosus* isolates positive for *aprB2* were also *aprB2* positive by genomic analysis. A total of 55/68 (81%) isolates positive for *aprB2* originated from Sweden and Norway with *aprB2* being the variant identified most frequently in these two countries (**Table 5.2** & **Figure 5.5**).

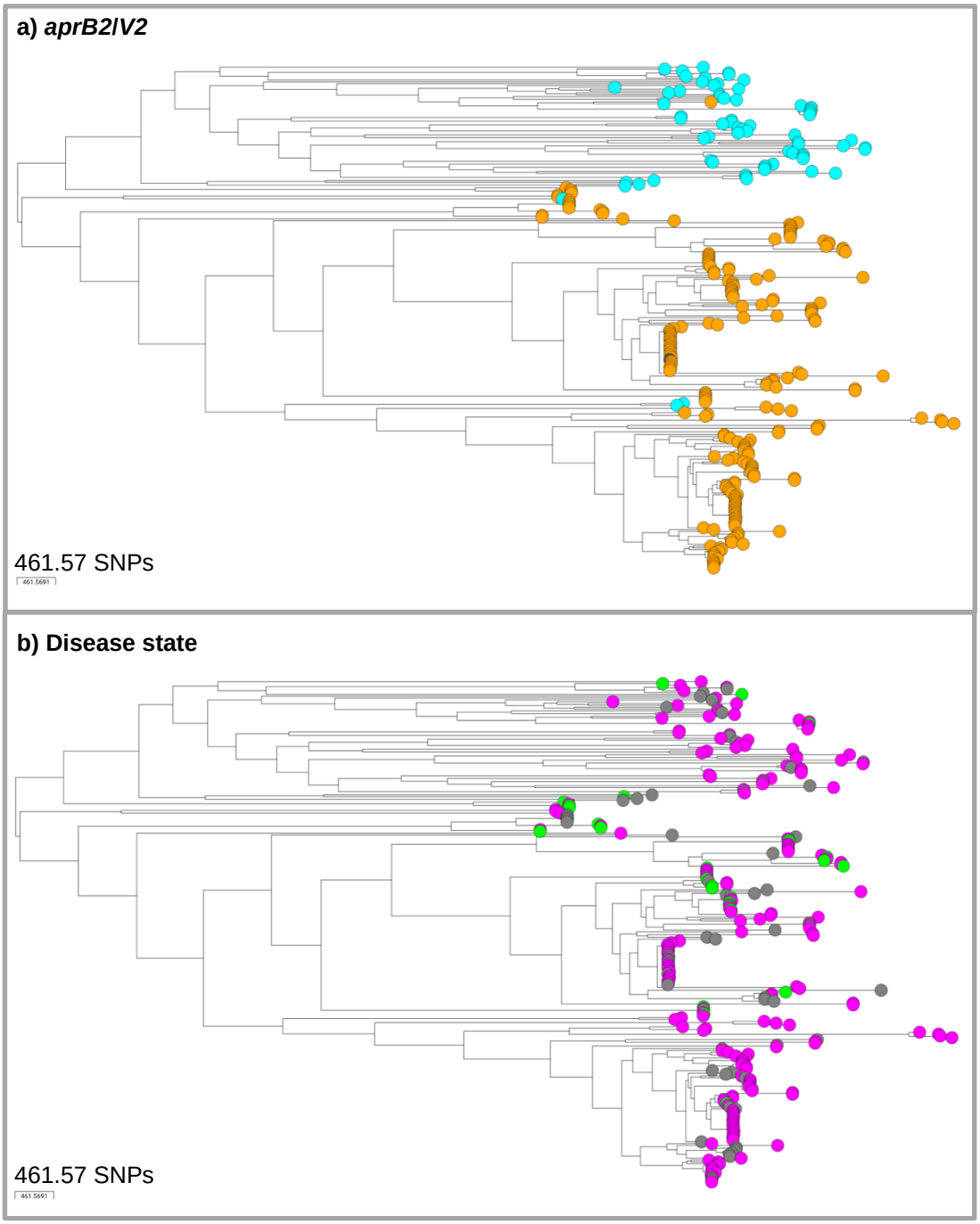


Figure 4.25: Maximum likelihood SNP tree of 275 *D. nodosus* isolates plus the reference *D. nodosus* VCS1703A which is positive for *aprV2* with recombination removed and rooted using *TempEst* 'best fitting root', isolates coloured by acidic protease from genomic analysis (a) and disease state (b).

a: *turquoise*: *aprB2*, *orange*: *aprV2*, *grey*: unknown. **b**: *lime green*: healthy, *pink*: ID and/or SFR, *grey*: unknown.

Table 4.31: Acidic protease identification by country from genomic analysis of 275 globally sourced *D. nodosus* isolates.

Country	No. of samples	No. (%) *aprB2* positive	No. (%) *aprV2* positive	Acidic protease unknown
UK	107	4 (1)	103 (99)	0
Norway	52	27 (52)	24 (46)	1 (2)
Australia	40	8 (20)	32 (80)	0
Sweden	34	28 (82)	6 (18)	0
Germany	33	0	33 (100)	0
Denmark	6	0	6 (100)	0
Bhutan	1	1	0	0
India	1	0	1	0
Nepal	1	0	1	0

A total of 47/51 (92.2%) *D. nodosus* isolates positive for *aprB2* where disease state was known were from feet with ID and or SFR (**Table 5.3**). A total of 138/161 (85.7%) *D. nodosus* isolates positive for *aprV2* where disease state was known were from feet with ID and or SFR (**Table 5.3**). The branch highlighted in bold in **Figure 5.5 a & b** showed divergence between two *D. nodosus* isolates from Australia, one with *aprB2* identified and the other with *aprV2* identified.

Table 4.32: Number and percentage of samples positive for *aprB2/V2* by disease state of the 275 *D. nodosus* isolates with acidic protease known by genomic analysis.

	DISEASE STATE			
Acidic protease	**Healthy**	**Footrot (ID and/or SFR)**	**Unknown**	**Total**
B2	4 (14.8)	47 (25.4)	17 (27.0)	68
V2	23 (85.2)	138 (74.6)	46 (73.0)	207
Total	27	185	63	275

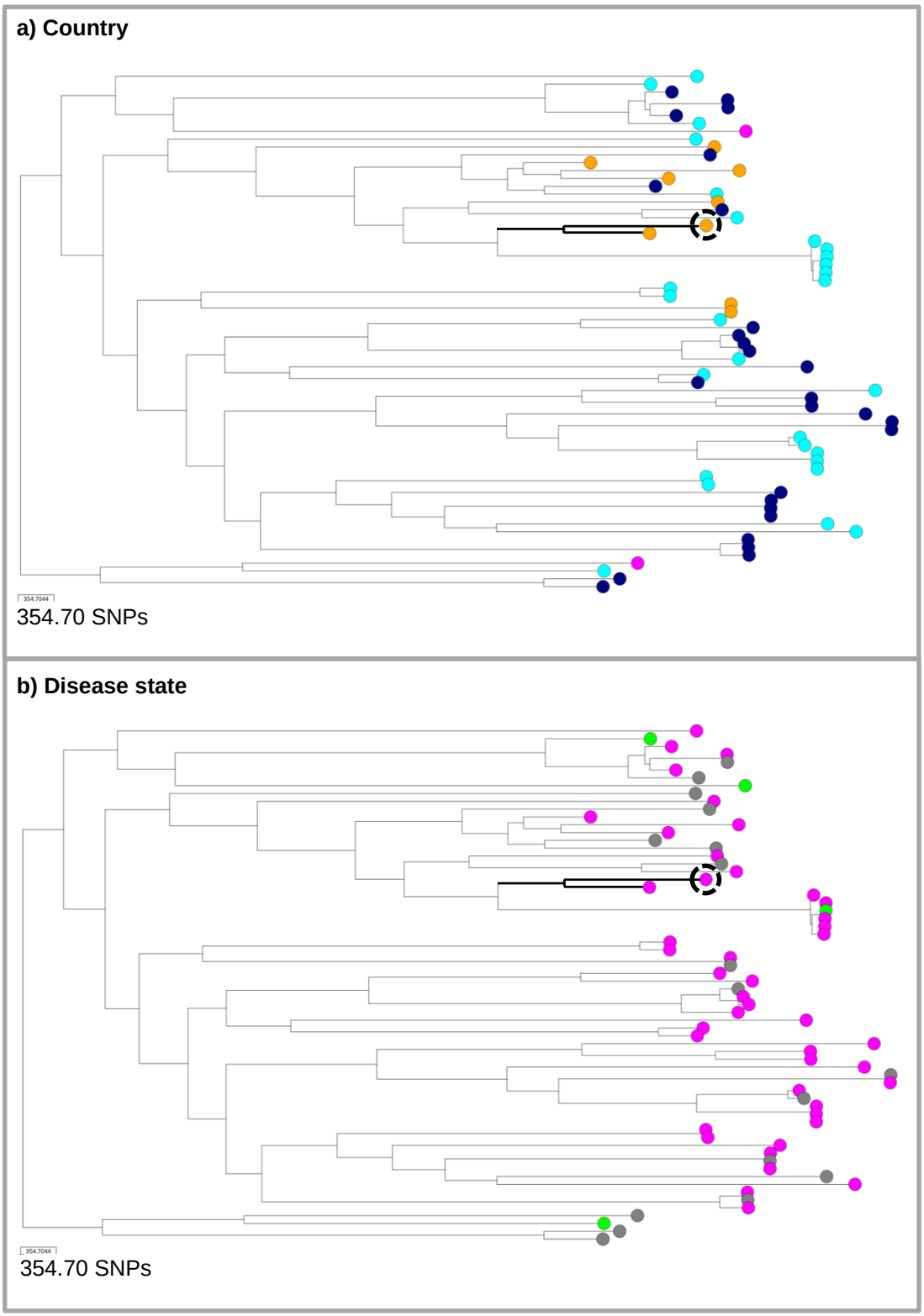


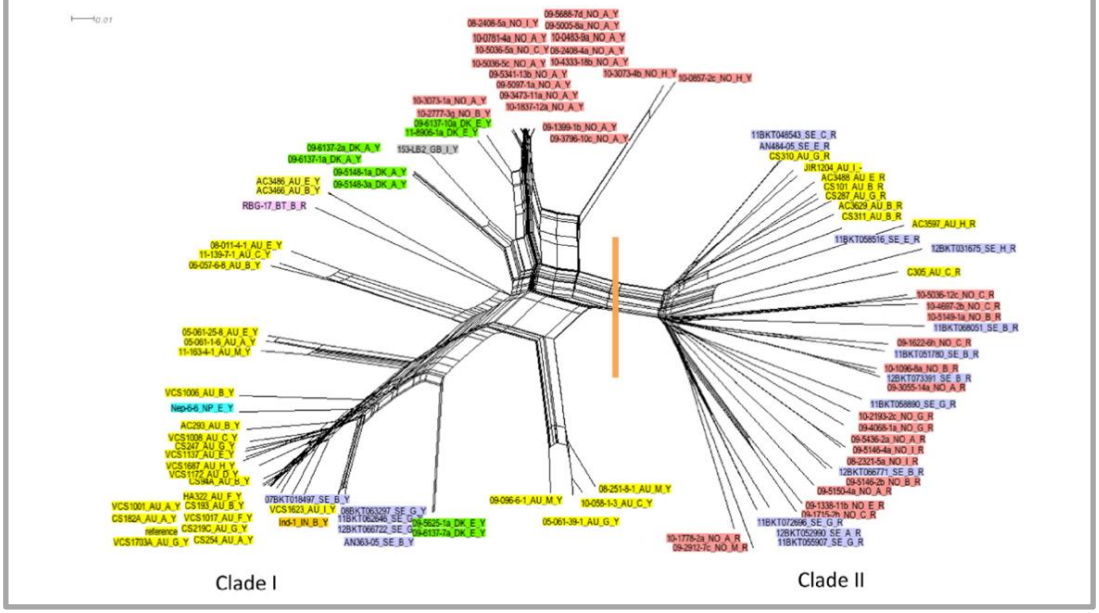
Figure 4.26: Subtree from top branch of Figure 5.4a of *D. nodosus* isolates all positive for *aprB2* except one from Australia positive for *aprV2* (black dashed outline) coloured by country (a) and by disease state (b).

a: *navy*: Norway, *turquoise*: Sweden, *orange*: Australia, *pink*: UK. **b**: *lime green*: healthy, *pink*: ID and/or SFR, *grey*: unknown.

4.4.4 Comparison of global tree produced in the current study and that produced by Kennan *et al.* (2014)

The tree produced in the current study in **Figure 5.4a** was compared to the tree produced by Kennan *et al.* (2014) and this is shown in **Figure 5.6**. In the tree from the current study the majority of isolates that were positive for *aprB2* were clustered together as in the Kennan *et al.* (2014) tree. However, isolates outside of this cluster were also positive for *aprB2* contrary to the Kennan *et al.* (2014) tree. Furthermore, some isolates amongst the *aprB2* cluster were positive for *aprV2*.

a) Kennan *et al.* (2014) 103 *D. nodosus* isolates, clade 1 – *aprV2*, clade 2 – *aprB2*



b) Current study 275 *D. nodosus* isolates coloured by acidic protease (includes Kennan *et al.* (2014) 103 isolates)

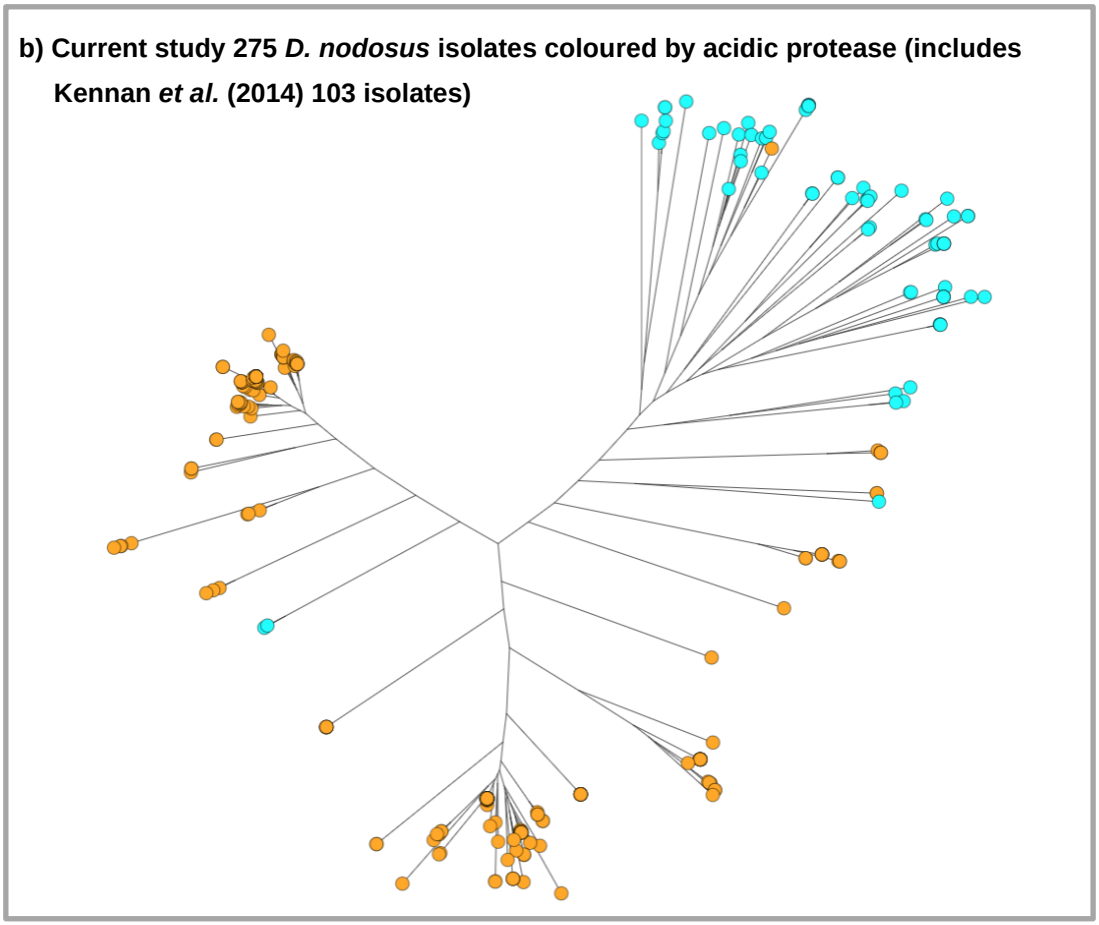


Figure 4.27: Comparison of the network diagram from Kennan *et al.* (2014) showing 103 *D. nodosus* isolates (a) and the maximum likelihood SNP tree of 275 *D. nodosus* isolates (b) that was shown in Figure 5.2.

a: *yellow*: Australia, *red*: Norway, *lilac*: Sweden, *green*: Denmark, *pink*: Bhutan, *blue*: Nepal, *orange*: India, *grey*: UK. **b**: *turquoise*: *AprB2*, *orange*: *aprV2.*

4.4.5 Genomic analysis of the *fimA* gene to identify the serogroups in the globally sourced *Dichelobacter nodosus* isolates

This section used the group of 275 global *D. nodosus* isolates with representative isolates from the Bristol farm. All 10 known serogroups were identified in the globally sourced isolates through laboratory and genomic analysis. The gene search script yielded top serogroup percentage nucleotide identities for each isolate at > 83%. Laboratory analysis was available for 187/275 *D. nodosus* isolates and genomic analysis yielded serogroup identification in 273/275 *D. nodosus* isolates (**Table 5.4**). Both laboratory and genomic serogroup identification was available for 186 *D. nodosus* isolates. Serogroup identification by laboratory and genomic analysis matched in 125 (67.2%) *D. nodosus* isolates. Serogroup identification from genomic analysis is given in **Table 5.5** and **Figure 5.6**, with no phylogenetic pattern in serogroup identification indicated.

Table 4.33: Number of serogroups identified through laboratory and genomic analysis of 275 *D. nodosus* isolates.

Serogroup	No. identified by lab analysis	No. identified by genomic analysis	No. where lab & genomic analysis matched
A	46	11	5
B	48	61	44
C	14	24	14
D	5	35	4
E	16	22	13
F	2	12	2
G	15	19	12
H	22	27	18
I	14	60	11
M	4	2	2
B & H	1	0	0
TOTAL	187	273	125
NI/NA	105	3	-

NI/NA: Isolates where no serogroup was identified / the isolate did not undergo serogroup analysis.

Table 4.34: Numbers of each serogroup identified through genomic analysis of 275 *D. nodosus* isolates.

	NO. OF SAMPLES DETECTED BY SEROGROUP (A – M)											
Country	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**M**	**To**	**NI/NA**
UK	1	36	9	13	2	6		16	24		107	
Norway	7	5	5		4	2	4	4	21		52	
Australia	3	9	7		4	2	5	2	5	2	40	1
Sweden		7	3		3		10	3	7		34	1
Germany		2		22	4	2		2	1		33	
Denmark					4				2		6	
Bhutan		1									1	
India		1									1	
Nepal					1						1	

To: Total number of serogroups.
NI/NA: Isolates where no serogroup was identified / the isolate did not undergo serogroup analysis.

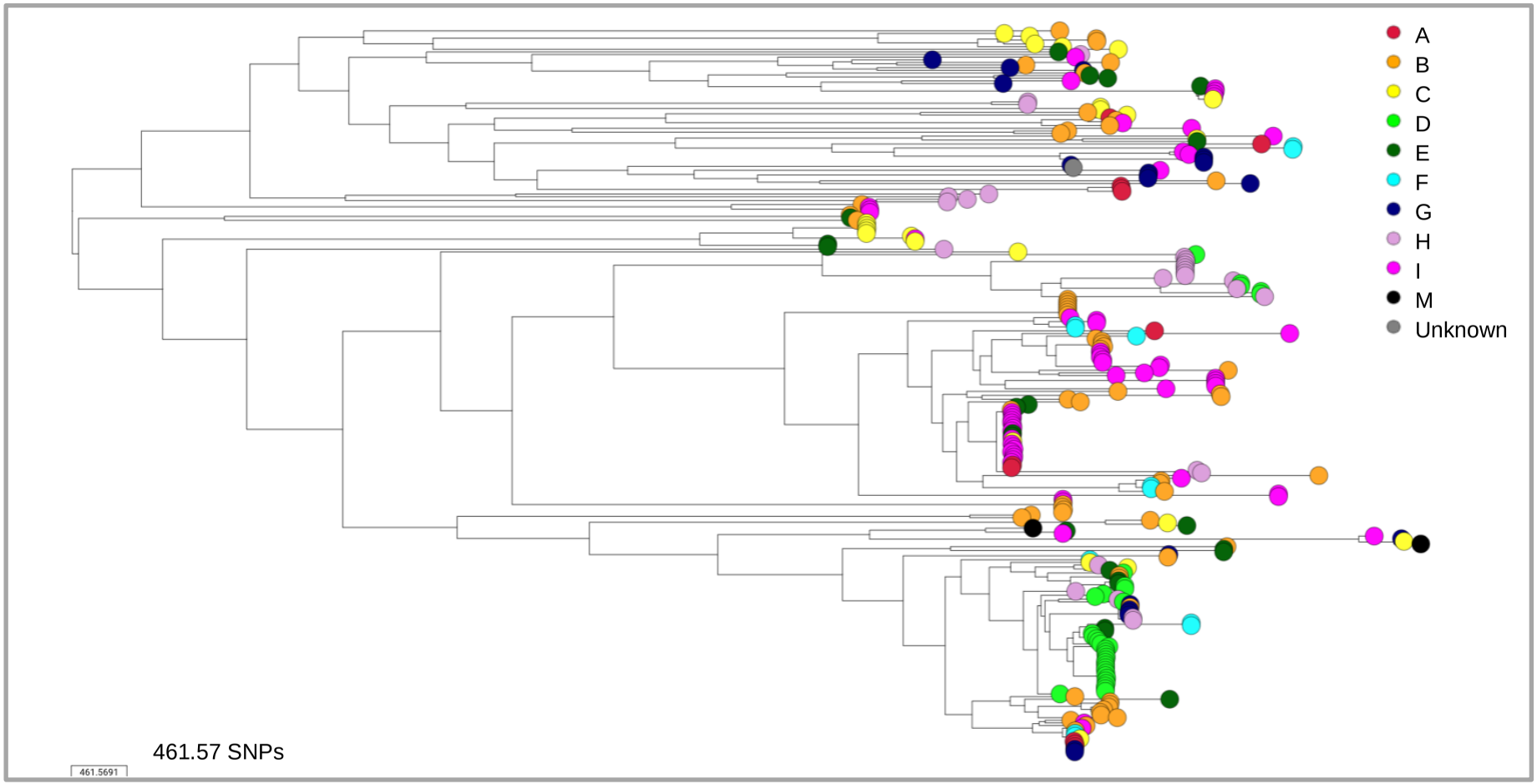


Figure 4.28: Maximum likelihood SNP tree of 275 *D. nodosus* isolates plus the mapping reference *D. nodosus* VCS1703A with recombination removed and rooted using *TempEst* 'best fitting root', isolates coloured by serogroup identified through genomic analysis.

4.4.6 Recombination

The high frequency of recombination events occurring in *D. nodosus* is demonstrated in **Figure 5.7**, with peaks in recombination occurring in the gene regions of *fimA*, *fimB*, *omp1A*, *omp1D*, *omp1E*, plus an unannotated gene highlighted in **Figure 5.7**. Recombination rates (r/m) and the probability of a site being altered by recombination or mutation (ρ/θ) are given for each Bristol clade in **Table 5.6**.

Table 4.35: Number of SNPs detected before and after recombination was removed.

No. of samples	Total no. of SNPs	Total no. of vertically inherited SNPs	Total r/m	Mean r/m	Total ρ/θ	Mean ρ/θ
275	344956	207991 (60.3%)	1480.38	2.70	73.00	0.13

r/m: Number of homologous SNPs in homologous recombination regions per vertically inherited SNP (mutation).
ρ/θ: Number of homologous recombination events per vertically inherited SNP (mutation).

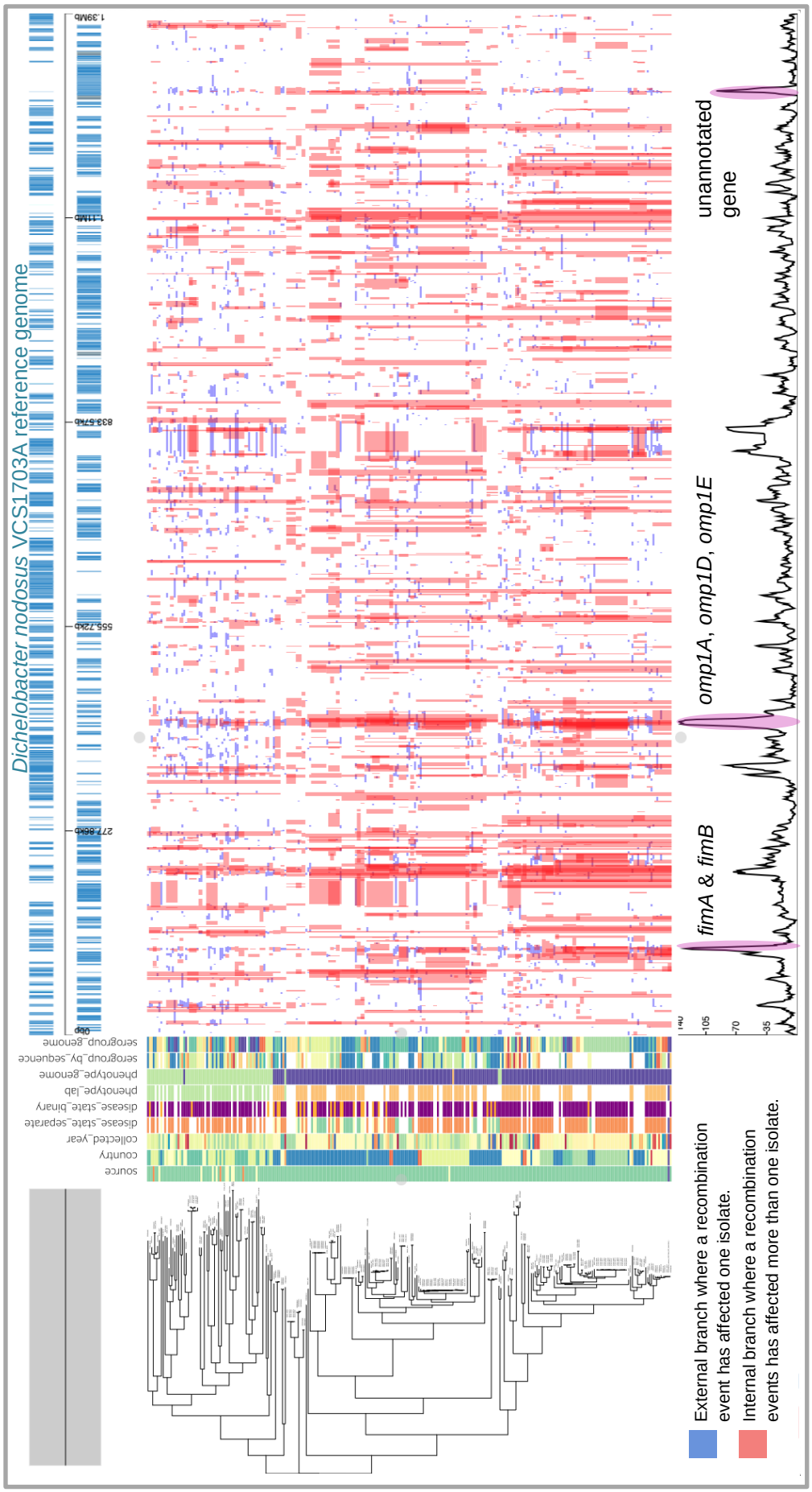


Figure 4.29: Recombination predictions along 1.39Mb from the maximum likelihood SNP tree of 275 *D. nodosus* isolates with peaks in recombination highlighted and identified through searching the reference genome.

BEEF & LAMB

5 Discussion

5.1 **Detection of *Dichelobacter nodosus* and associations between load and the severity of footrot**: Bristol Study flock samples

No studies have determined the load of *D. nodosus* by lesion severity score before. The first key result from the current study was the increase in mean *D. nodosus* load with each score of increasing severity of ID (**Table 2.16**). This indicates that *D. nodosus* load on feet which is higher than on healthy feet is indicative of more severe ID, a greater area affected. This is similar to conclusions from Witcomb *et al.* (2014) who observed an increase in the load of *D. nodosus* prior to and during ID. The results from the current study highlight the close relationship between ID lesion severity and load. Furthermore, the lack of association between load and low scoring severe footrot, indicate that ID is the infectious stage of footrot.

The current results also demonstrate for the first time that a larger proportion of feet with ID1 compared with other ID scores were negative for *D. nodosus*. The greatest increase in *D. nodosus* detection and load was observed between ID1 and ID2 (**Table 2.17**). This is unlikely to be because of low sensitivity of detection because the qPCR used detects to one *D. nodosus* bacterium per microlitre (Calvo-Bado *et al.*, 2011b). It is more likely a misclassification in phenotype such that ID1 is not always ID. It is highly likely that sheep will have mild irritation if the interdigital skin that phenotypically is indistinguishable from initial ID. Particularly as this is seen as part of the conditions necessary for *D. nodosus* to cause footrot (Beveridge, 1941, Egerton and Roberts, 1969). Models of disease have improved with ID scores of 1 being coded as 'healthy' rather than 'diseased' (Atia *et al.*, 2017, Clifton *et al.*, 2019). A flock with sheep with only ID score 1 might be less likely to have *D. nodosus* present on the farm than if more severe lesions are present. But for prevention of footrot, testing of the lesions would still be the best way of determining absence of *D. nodosus*.

The next key result was the reduction in load after treatment with FTA or PTA but not in sheep given only parenteral antibacterial treatment and no topical treatment. There was a strong similarity in reduction in load after treatment between treatment with FTA and treatment with PTA. Samples from diseased feet had higher numbers of *D. nodosus* cells and mean *D. nodosus* load was significantly reduced after treatment with FTA or PTA. This, along with the lack of a reduction in mean *D. nodosus* load in sheep without footrot after treatment with parenteral antibiotics and no topical treatment (**Figure 2.8**), suggests that the antibiotic foot spray reduces *D. nodosus* load on the interdigital skin. As implied by the first key result, a reduction in load indicates reduced disease and would also reduce the spread of *D. nodosus* and chances of disease developing in other sheep. Disease could be resolved sooner if the ewe was treated at ID1. As seen in Kaler *et al.* (2010a), sheep with a

locomotion score ≤ 2 recovered sooner than those treated with locomotion score 3. There were only six samples from occasions where parenteral antibiotics was given but no antibiotic foot spray was given hence the larger standard errors. Both designated treatments were effective in reducing positive scores for lameness and footrot lesions for the majority of ewes treated but, FTA yielded a greater reduction in footrot lesions than PTA.

The aim of the Smith *et al.* (2014) study was not to compare footrot treatments and so data on foot trimming only for example was not taken. However, we know from Kaler *et al.* (2010a), Wassink *et al.* (2010), Kaler and Green (2009) and Green *et al.* (2007) that lameness and lesions recover faster and more effectively when given parenteral and topical antibiotics as opposed to a method of treatment involving foot trimming. Wassink *et al.* (2003) highlighted the independently beneficial effects of both parenteral and topical treatment of footrot. From the current study, topical antibiotic treatment is indicated to be key in reducing the spread of *D. nodosus*.

The third key result was determining that *D. nodosus* qPCR positive and culture positive interdigital skin samples have a significantly higher mean *D. nodosus* load than *D. nodosus* qPCR positive but culture negative interdigital skin samples. Also, finding that 21% of *D. nodosus* qPCR positive but culture negative interdigital skin samples could be detected using qPCR but would unlikely be detected through culturing changes our view on appropriate detection methods. Culturing has been the most common method of identifying *D. nodosus* but this study shows that detecting *D. nodosus* from culturing alone would not give as true a representation of *D. nodosus* presence as previously thought (Locher *et al.*, 2018). This concurs with detection results from Frosth *et al.* (2012) who found real-time PCR to be three times more sensitive at detecting *D. nodosus* than culturing.

In this study, as previously found in Kaler (2008), locomotion score was not always representative of disease state. Almost half of routine samples from ewes showing no signs of lameness had a positive ID and/or SFR score. This implies that locomotion score is not always an accurate measure of footrot present. A lot of disease may be missed if locomotion score was solely used to judge foot health. Pain thresholds between sheep are likely to vary and so some sheep may not show signs of lameness but are still harbouring disease (Ley *et al.*, 1995). A limitation from the Smith *et al.* (2014) study was that no distinction was made between lesions newly emerging and established lesions healing. Positive detection of *D. nodosus* is less likely in inactive lesions than active lesions (McPherson *et al.*, 2018). This information could have aided the analysis of the effect of treatment on lesions more accurately. Lesion activity should be included in future assessments of lesions and lameness.

Although there was no significant difference in *D. nodosus* detection between interdigital skin and non-interdigital lesion samples, interdigital skin samples had a significantly higher mean *D. nodosus* load than non-interdigital lesion samples (**Table 2.18**). This links with *D. nodosus* load increasing with ID score with the interdigital skin harbouring higher counts of *D. nodosus*. Also, there were more than 10 times the number of interdigital skin samples collected than non-interdigital lesion samples. Significant results were established in non-interdigital lesion samples in mean *D. nodosus* load by ID score for example, but more lesion samples would have added to these results. Nevertheless, valid comparable results were obtained.

The numbers of cases of lameness for the subset of 25 ewes was not significantly different to that of the remaining 66 ewes of the Bristol study flock (**Table 2.4**). This gives confidence in the subset being representative of what would have been found had the whole flock been studied in such detail. The mean *D. nodosus* load of samples extracted by the three different extractors at different time points were also not dissimilar. The significant difference in mean $\log_{10}$(*D. nodosus* load +1) between routine and targeted sampling periods in the multivariable LM could be attributed to the significant association between mean $\log_{10}$(*D. nodosus* load +1) of locomotion scores 3 and 4 and targeted sampling periods. There was no significant difference in mean $\log_{10}$(*D. nodosus* load +1) between sampling weeks when tested as a single factor. These points add to the robustness of the methods carried out in the 2010-2011 Bristol study and analyses carried out in the current study. Further to the relationship between *D. nodosus* load and the severity of footrot, a limitation from this study was that only SFR scores 0 and 1 (very mild under-running of the hoof horn) could be analysed. With only three samples with SFR score 2, these were assigned SFR score 1. This could have impacted on the finding of no relationship between *D. nodosus* load and increasing SFR score, contrary to that found with increasing ID score.

In conclusion, an increase in *D. nodosus* load is associated with an increase in the severity of ID. Mean *D. nodosus* load was significantly reduced after treatment with FTA or PTA but not with parenteral antibiotics and no topical treatment. This suggests the action of the antibiotic foot spray in reducing *D. nodosus* load on the interdigital skin making it an essential part of footrot treatment. Finally, *D. nodosus* from culturing alone would not give as true a representation of *D. nodosus* presence.

5.2 Key virulence factors, *aprB2*, *aprV2* and *fimA* of *Dichelobacter nodosus* and associations with the severity of footrot: Bristol study samples

Both the 409 swab DNA samples and the 268 *D. nodosus* isolate DNA samples from Smith *et al.* (2017) provided a large dataset with which to assess serogroup detection in the Bristol study flock. The same four serogroups were identified in both isolate DNA and swab DNA analysis. This strongly suggested that these were the only serogroups present in this flock. At a flock level, isolate analysis was sensitive enough to detect all the serogroups, but swab DNA gave greater and more accurate quantitative serogroup data than isolate DNA. There was a significant association between serogroup detection and type of analysis. Isolate DNA (multiplex PCR) and swab DNA (singleplex PCR) detected similar proportions of serogroup B. However, the proportion of samples positive for the remaining three serogroups were higher in swab DNA than isolate DNA. For serogroup H in particular, swab DNA yielded more than nine times the proportion positive than isolate DNA (I more than two times the proportion positive, D more than four times the proportion positive). This could have been for one of two reasons. The Bristol study isolate DNA analysed in Smith *et al.* (2017) was serogroup tested by multiplex PCR whereas singleplex PCR was used on swab DNA in the current study. Competition between primers is a risk with multiplex PCR and so could have affected detection of serogroups in isolate DNA (Dhungyel *et al.*, 2002). Alternatively, *D. nodosus* isolate culture may have been less favourable for serogroups H, I and D, which could have been outcompeted by growth of serogroup B.

Where both swab DNA and isolate DNA data were available for a sample, in nearly three quarters of these samples the same serogroup was detected in isolate DNA and swab DNA analysis but additional serogroups could be detected in swab DNA. More than three quarters of samples where the same serogroup was detected in isolate DNA and swab DNA analysis but additional serogroups could be detected in swab DNA, serogroup H was the additional serogroup. This indicates that serogroup H is less detectable by culture. This sample size was fairly small, however in only 1/31 interdigital skin samples did swab DNA and isolate DNA serogroup detection differ. This gives confidence in both sources providing accurate data but, as shown in **Chapter 2**, non-culture-based swab DNA analysis was more sensitive and therefore provides a more accurate representation of *D. nodosus* present. Serogroups B and H were most commonly detected and appeared to persist on feet over time.

All four serogroups could be detected across the range of ID and SFR scores recorded. There was no indication of any one serogroup causing more severe lesions. There were not enough samples with treatment and post treatment data and serogroup detection in this study to analyse the effect of treatment on serogroup detection. If the quantity of each serogroup present could have been

measured using quantitative PCR this might have given an indication of whether serogroup presence is affected by treatment. No association was found between serogroup strain detected and ID or SFR score. This could be due to two serogroups dominating with the range of disease severities occurring.

In conclusion, the same four serogroups were identified through isolate DNA and swab DNA analysis but swab DNA gives greater and more accurate quantitative serogroup data than isolate DNA. No association was identified between serogroup detection and ID or SFR severity.

5.3 Key virulence factors and phylogenetic diversity of *Dichelobacter nodosus* and associations with the severity of footrot: On a single UK farm

A significant temporal signal was identified in clade 1 (**Figure 4.11**) with accurate approximation indicated by the confidence intervals and parameter traces (**Figures 4.15** and **4.16**). The most recent common ancestor was predicted to have emerged in 1998, the same year new stock was introduced onto the farm for research purposes having previously been a closed flock. A rate of 14 substitutions/mutations per year occurred since. Three distinct clades identified from the Smith *et al.* (2017) study isolates (**Figure 4.2**) could suggest three introductions of *D. nodosus*. The last entry of new stock were three rams, one from Bangor North Wales, and two from different farms in Somerset. These could be additional sources of *D. nodosus* strain divergence in the flock. However, the *D. nodosus* phylogeny in the flock prior to the ram introductions was not known. Further temporal analysis could be done on clades 2 and 3 to see if a significant temporal signal may be found through BactDating. The mutation rate in relation to resulting diversity could also be investigated. New stock are a common source of new strains and infection and so this highlights the importance of having biosecurity measures including quarantine and assessing and disinfecting any new sheep brought in to minimise the spread of *D. nodosus* and outbreaks of footrot.

The same four serogroups, B, D, H, and I, were identified on the Bristol farm through both laboratory and genomic analysis. Although serogroups B and I were identified in clades 2 and 3, the phylogeny of clade 2 was closer to that of clade 1, identified with serogroups D and H, than clade 3. There was considerable genetic divergence in *D. nodosus* isolates in all three clades (**Figure 4.3**). Although only two known serogroups were identified in each clade, all three clades exhibited the entire range of ID and SFR scores recorded. Serogroups A, B, C, E, F, G, I and M and serogroups D and H have been linked with virulent and benign strains respectively (Kennan *et al.*, 2011, Kennan *et al.*, 2014, Claxton *et al.*, 1983). But the range of disease presentations shown for serogroups D and H in the Bristol flock suggest they are unlikely to be benign. Interestingly, serogroups D and H were the predominant serogroups identified in a recent study of sheep in an area of Brazil (de Carvalho *et al.*,

2018). They also identified mixed infections of D + H + I, and D + H. This, along with Kennan *et al.*, 2011, indicates that serogroups D and H are often found together. Phylogenetic analysis revealed no clear associations between serogroup identification by genomic analysis and ID score or SFR score or whether the sheep was a ewe or a lamb in any of the three clades. *D. nodosus* strains regardless of serogroup are able to cause footrot.

There were a considerable number of recombination events occurring across the majority of isolates. With mean r/m recombination rates of 11.36, 5.75 and 4.56 for clades 1, 2 and 3 respectively. Also, the ratio of recombination events to mutation events (ρ/θ) of 0.66, 0.31 and 0.21 for clades 1, 2 and 3 respectively indicates that more mutations are occurring than recombination events. These are not dissimilar to those found in *Pseudomonas aeruginosa* with a ρ/θ value of 0.181, also a Gram-negative anaerobe with a twitching motility mechanism (Dettman *et al.*, 2015). *Escherichia coli*, another Gram-negative anaerobe, exhibits a ρ/θ ratio of 1.024; mutations occurring as often as recombination (Didelot *et al.*, 2012). Recombination predictions in **Figure 4.4** showed differing recombination events between serogroups within clades. This adds to the indication of genomic serogroup identification being accurate.

The key peaks in recombination were found for the gene regions of *fimA*, *fimB*, *omp1A* and an un-annotated gene region. As *fimA* is the first factor used to classify the 10 known serogroups this is likely to be a key peak of recombination (Kennan *et al.*, 2011, Myers *et al.*, 2007). The *omp1* gene has been observed to frequently switch between variants which are highly divergent hence the high frequency of recombination (Moses *et al.*, 1995, Myers *et al.*, 2007). The *omp1* gene has been linked with antigenic variation and may divert host immune responses and as such is linked with virulence of *D. nodosus* (Moses *et al.*, 1995). However, no recent studies on *omp1* in *D. nodosus* have been carried out. Both the fimbrial and *omp1* gene regions would be worth investigating further to determine any associations with virulence or phylogeny of *D. nodosus*.

Genomic identification of serogroups was coherent with phylogenetic diversity in all three clades. Whereas laboratory identification was intermittent and less coherent with phylogenetic diversity in all three clades. In 190/192 *D. nodosus* isolates where laboratory and genomic serogroup identification matched, percentage nucleotide identity to the serogroup reference from genomic analysis was more than 95%. This along with the coherence of the genomic analysis, and the 82.8% match with laboratory analysis, indicated genomic serogroup identification shows greater accuracy. The most distant branch in each clade was identified as a different serogroup. This could be as a result of serogroup conversion occurring from H to D in clade 1 and B to I in clades 2 and 3 (Kennan *et al.*, 2003, Gilhuus *et al.*, 2014). This adds to the evidence for serogroups D and H in particular which

appear to often be found together (Kennan *et al.*, 2011, de Carvalho *et al.*, 2018) which may be as a result of serogroup conversion. Further investigation of the phylogeny of the fimbrial gene region could help elucidate common ancestors between serogroups. This would give greater knowledge in developing a more targeted and effective vaccine formulation.

In conclusion, the most recent common ancestor identified in one of the three Bristol farm clades was predicted to have emerged in 1998. This was the same year new stock was introduced onto the farm for research purposes having previously been a closed flock. The same four serogroups, B, D, H, and I, were identified on the Bristol farm through both laboratory and genomic analysis. This gives further evidence that these are the only serogroups present in this flock. Phylogenetic analysis revealed no clear associations between serogroup identification by genomic analysis and ID score or SFR score in any of the three clades. *D. nodosus* strains, regardless of serogroup, are able to cause footrot.

5.4 Key virulence factors and phylogenetic diversity of *Dichelobacter nodosus* and associations with the severity of footrot: Globally sourced isolates

Analysis in this chapter was focused primarily on the two key factors linked with virulence of *D. nodosus*; acidic protease *B2*/*V2* and *fimA*-determined serogroup. This furthered the knowledge around these two factors regarding disease state and gives new insights into what is of use in controlling this endemic disease.

Acidic protease identification was identical between laboratory and genomic analysis in all but one *D. nodosus* isolate. Moreover, with more than two thirds of serogroup detection between laboratory and genomic analysis matching, genomic identification was used for further analysis of serogroup and acidic protease detection.

Nearly all *D. nodosus* isolates positive for *aprB2* were taken from feet with a form of footrot. Also, *aprV2* was detected in more than 85% of *D. nodosus* isolates taken from clinically healthy feet. Acidic protease genotype did not associate with being healthy or having a form of footrot. This is in contrast to *aprB2* being associated with interdigital dermatitis, suggested as the benign, non-progressive, form of footrot (Stäuble *et al.*, 2014, Riffkin *et al.*, 1995). Acidic protease *V2* has been associated with the virulent underrunning of the hoof (Riffkin *et al.*, 1995) through degradation of the hoof horn (Kennan *et al.*, 2010). But both *aprB2* and *aprV2* have since been observed to induce a host inflammatory response (Maboni *et al.*, 2017). The results from the current study suggest that *aprB2* is found on feet with the more severe form of the disease contrary to the view of being benign. The scoring of footrot lesions does differ between countries where often one scale is used incorporating

ID and SFR (Egerton and Roberts, 1971). This may have an impact on the comparison of acidic protease genotype with disease state at the earlier signs of disease progressing (Foddai *et al.*, 2012).

This study also showed that, phylogenetically, isolates cannot be distinguished by acidic protease detection alone. **Figure 5.6** showed similar clustering of *aprB2* positive isolates but they were not distinct from *aprV2* isolates. In the subtree of isolates sourced predominantly from Sweden and Norway with all but one being positive for *aprB2* (**Figure 5.5**), the branch highlighted included an *aprB2* isolate and the one *aprV2* isolate in this subtree. This suggests the single amino acid switch between *aprV2* (tyrosine) and *aprB2* (arginine) (Riffkin *et al.*, 1995) can occur sporadically. The majority of *D. nodosus* isolates positive for *aprB2* coming from Sweden and Norway where *aprB2* dominates suggests the acidic protease genotype is simply mirroring the phylogeographical structure of *D. nodosus*. This is similar to Blanchard *et al.* (2018) who found strain type to correlate with country of origin.

The analyses in this chapter show that neither serogroup nor acidic protease genotype correlated with foot disease state. Neither did serogroup and acidic protease genotype correlate with each other. This indicates that identifying neither acidic protease genotype nor serogroup would actually help in devising strategies to control of footrot.

D. nodosus has been reported to be highly recombinant (Russell *et al.*, 2014) and the current data set confirms this (**Figure 5.8**). A high frequency of recombination along with clonal expansion has led to the rapid evolution of *D. nodosus* within mixed populations (Russell *et al.*, 2014, Buller *et al.*, 2010, Gilhuus *et al.*, 2014, Smith *et al.*, 2017). This is indicated in the current study where phylogenetic clades appear to have crossed between neighbouring countries Sweden and Norway (**Figure 5.5**). The key peaks in recombination were found for the gene regions of *fimA*, *fimB*, *omp1A*, *omp1D*, *omp1E* and an un-annotated gene region. As mentioned in **Chapter 4**, the fimbrial and *omp*1 gene regions are documented sites of recombination (Kennan *et al.*, 2011, Myers *et al.*, 2007, Moses *et al.*, 1995). *FimA* encodes the main fimbrial subunit, is needed for cell adhesion and twitching motility, and is the first factor used to classify the 10 known serogroups (Kennan *et al.*, 2011, Myers *et al.*, 2007, Kennan *et al.*, 2014). The *omp1* gene has been linked with antigenic variation and may divert host immune responses (Moses *et al.*, 1995). Both the fimbrial and *omp1* gene regions would be worth investigating further to determine any associations with virulence of *D. nodosus*.

There were not enough isolates from sheep, cows or goats to analyse but there was no indication of a phylogenetic distinction (**Figure 5.3**). Previous studies have shown cows and goats can also be reservoirs of *D. nodosus* (Ghimire *et al.*, 1996, Belloy *et al.*, 2007, Wani *et al.*, 2015, Osova *et al.*,

2018). This could impact the control of *D. nodosus*, and therefore footrot, in mixed-species pastures with cross-infection between cattle and sheep previously identified (Knappe-Poindecker *et al.*, 2014, Rogdo *et al.*, 2012).

A total of 567 high quality sequences were obtained from the 661 *D. nodosus* isolates submitted for whole genome sequencing. This reflects very positively on the collection and processing of these isolates. A median total sequence length of 1316417bp is shorter than the reference genome of 1.39Mb however a median genome coverage of 93.65% and median number of contigs of 16 suggests high quality assemblies. A limitation of this work was that the disease severity score given for the foot from which isolates were obtained was often unavailable. More detail on the metadata of isolates could have aided comparisons with disease severity. Another limitation indicated by the work in **Chapter 2**, **section 2.3.5.3** is that genomic sequences were obtained from *D. nodosus* isolates which have shown to be less representative of *D. nodosus* than direct swab analysis. The use of metagenomic analysis could provide greater detail if such techniques could be refined to identify high quality assemblies from mixed foot swabs carrying such a diverse range of bacterial species. Metagenomics would then be a highly useful tool for future phylogenetic analyses having been used successfully in analysis of bacterial phyla from bovine digital dermatitis samples (Zinicola *et al.*, 2015). Furthermore, a broader spectrum of *D. nodosus* isolates from the countries analysed and other countries with footrot not sampled, like Brazil and India, would be useful in further investigation of associations between the fimbrial and *omp1* gene regions and pathogenicity (de Carvalho *et al.*, 2018).

In conclusion, isolates that are positive for either acidic protease V2 or B2 exhibit the range of disease presentations and their detection appears to reflect their geographical location. Both the fimbrial and *omp1* gene regions could be investigated further as markers of virulence of *D. nodosus*.

6 General discussion and industry messages

The majority of the work in this study was focused around analysis of swab DNA and isolate DNA taken from the feet of a flock of 91 ewes from one farm studied in detail previously over 10 months. This work brought to light new knowledge around *D. nodosus* load and the severity of footrot, how *D. nodosus* load is affected by treatment, and differences in methods of detecting *D. nodosus*. The key virulence factors previously associated with clinical disease were then investigated both through laboratory and genomic analysis. These factors along with the phylogenetic diversity of *D. nodosus* were investigated on both a single farm scale and global scale.

From investigating the load of *D. nodosus* on the interdigital skin and non-interdigital lesions of ewes' feet in Chapter 2, mean load increased with increasing ID score. Also, a larger proportion of swab DNA samples from feet recorded as ID1 were negative, so ID1 may not always be ID. Both foot trimming plus topical antibiotic foot spray and parenteral antibiotics plus topical antibiotic foot spray yielded a reduction in mean *D. nodosus* load after treatment. This reduction in mean *D. nodosus* load was not seen on sheep without footrot after treatment with parenteral antibiotics and no topical treatment. This suggests that the topical antibiotic foot spray reduced *D. nodosus* load on the interdigital skin. Topical antibiotics are likely to reduce the spread of *D. nodosus* and so reduce the chances of further disease developing. The action of topical antibiotics are independently effective alongside using parenteral antibiotics and this is the most effective treatment of footrot (Wassink *et al.*, 2003, Kaler *et al.*, 2010b). The beneficial effect of using topical antibiotics to reduce the load of *D. nodosus* on feet was previously hypothesised but had not been tested. This study shows topical antibiotics to be a key part of the control of footrot.

When assessing footrot lesions, it is recommended that farmers turn sheep over and inspect their feet at gathering times and treat any signs of ID or SFR immediately. Almost half of routine samples in this study from ewes showing no signs of lameness had a positive ID and/or SFR score. If farmers purely use visible lameness this may not always be an accurate measure of footrot in their flock. A limitation in this study was that only SFR scores 0 and 1 (very mild under-running of the hoof horn) could be analysed. This could have impacted on the finding of no relationship between *D. nodosus* load and increasing SFR score, contrary to that found with increasing ID score. However, previous studies have indicated *D. nodosus* load on the surface decreases if disease progresses to severe footrot as *D. nodosus* destroys the epithelial layer and invades deeper tissue (Witcomb *et al.*, 2014, Witcomb *et al.*, 2015).

With regards to laboratory methodologies, mean *D. nodosus* load of swab DNA where isolates were successfully cultured was higher than where isolates were not obtained. In addition, more than one fifth of samples without isolates could be detected using quantitative PCR but would unlikely be detected through culturing. This shows that detecting *D. nodosus* from culturing alone gives a qualitative indication which could be sufficient to help in vaccine development for outbreaks of footrot. But does not give as true a representation of *D. nodosus* presence as direct quantitative DNA analysis for more in-depth analyses of *D. nodosus* pathogenicity. PCR based laboratory methods should be used in future analyses to ensure a more accurate detection rate (Frosth *et al.*, 2012).

In addition, swab DNA analysis yielded higher detection rates for serogroups H, I and D (H in particular) than isolate DNA analysis. More than three quarters of samples where additional

serogroups could be detected in swab DNA than isolate DNA, serogroup H was the additional serogroup. This indicates that serogroup H may be less detectable by culture from this flock. Possibly it was less resilient at growing in laboratory conditions. In only 1/31 interdigital skin samples did swab DNA and isolate DNA serogroup detection differ. This gave confidence in both sources providing accurate data but, as already indicated, non-culture-based swab DNA analysis was more sensitive and therefore provides a more accurate representation of *D. nodosus* present. Furthermore, the coherence of genomic serogroup analysis shown in the Bristol flock isolates, indicated genomic serogroup identification shows even greater accuracy than laboratory isolate analysis.

The isolates sequenced from the Bristol flock showed three phylogenetically distinct clades. Temporal signal was identified in clade 1 and the most recent common ancestor identified to date from 1998 when new stock was brought in to the previously closed flock. This gives further evidence to the importance of adequate biosecurity measures in minimising footrot in flocks. Quarantine of new and diseased sheep should be a top priority for farmers, insuring there is no spread of contact to current stock until the quarantine period of 4 weeks (ideally) is completed. All four feet of new sheep should be disinfected before being introduced to the current flock. The two remaining clades could have developed from the introduction of rams from different farms. This is commonly thought to be a source of new strains of *D. nodosus* but needs further research into the phylogeny of *D. nodosus* before and after ram introduction.

From genomic serogroup analysis of the Bristol flock, clade 1, the smallest clade, comprised serogroups D and H and the larger clades, 2 and 3, both comprised serogroups B and I. Yet phylogenetically clades 1 and 2 were more closely linked. Although only two known serogroups were identified in each clade, all three clades exhibited the entire range of ID and SFR scores recorded. So isolates that appear phylogenetically almost identical were identified across the range of disease severities. Moreover, no associations were found between serogroup identification by genomic analysis and ID or SFR score in the Bristol flock isolates. The same with laboratory identification of serogroups by singleplex PCR. All four serogroups caused footrot.

With regards to the current vaccine, it would be advisable for farmers not already using the vaccine to start using it biannually and monitor its effects. This should be done alongside current recommended management strategies for treatment of footrot. However, further research is needed in vaccine design as the variation in success rates (Winter *et al.*, 2015, Duncan *et al.*, 2012) could be attributed to the lack of association between serogroup (dominant or not) and disease state as indicated by this study.

From global *D. nodosus* isolate analysis where both aprB2 and aprV2 were identified, acidic protease identification was identical between laboratory and genomic analysis in all but one *D. nodosus* isolate. Accuracy of acidic protease identification appeared very strong, but these proteases did not correlate with disease presentation on the feet. Both genotypes were identified on healthy feet and feet with a form of footrot. The majority of *D. nodosus* isolates positive for aprB2 came from Sweden and Norway where aprB2 dominates suggesting the acidic protease genotype simply mirrors the phylogeographical structure of *D. nodosus*. This all indicates that all strains of *D. nodosus* regardless of acidic protease or serogroup are virulent and able to cause footrot. However, previous studies have shown that identifying serogroups present in a new outbreak of footrot in an area can then be used in targeted vaccine formulation which can help reduce the prevalence of footrot (Dhungyel *et al.*, 2002, John *et al.*, 1999).

In both the Bristol flock isolate analysis and globally sourced isolate analysis, considerable recombination was identified with more mutation events occurring than recombination events. In both analyses, peaks in recombination were found for the fimbrial and outer membrane protein 1 gene regions which may aid pathogenesis. The outer membrane protein region may shed more light on the virulence of *D. nodosus* and disease state and so should be investigated further.

6.1 Future work

Further investigation of the temporal signal and mutation rate in other two Bristol clades could provide further evidence of the introduction of the most recent common ancestor to the Bristol flock. Also, generation of phylogenetic trees of the fimbrial serogroups could help with clarifying distinctions between the serogroups. This could then help decipher any relationships with disease state and aid effective vaccine development. If metagenomic techniques could be refined for obtaining high quality assemblies of *D. nodosus* from mixed swab samples, this could give further detail to *D. nodosus* presence without relying on culture-based detection (Zinicola *et al.*, 2015). This could also allow for more detailed analysis of what other bacterial species co-exist with *D. nodosus* and how the quantities of each bacterium change with disease progression. A broader spectrum of *D. nodosus* isolates from the countries observed in this study and other countries with footrot not sampled, like Brazil and India, would be useful. This could aid further investigation of the fimbrial and omp1 gene regions and their relations to disease severity. As only mild SFR was observed in this study, analysis of a larger sample set of the range of SFR presentations may give more insight into changes in *D. nodosus* load by SFR severity score. It would also be interesting to look at whether there is inherent resistance to footrot by particular sheep in a flock. Genome analysis of any sheep identified with possible resistance traits compared with other sheep could help reveal what may be producing these resistance traits.

7 References

AGBAJE, M., RUTLAND, C. S., MABONI, G., BLANCHARD, A., BEXON, M., STEWART, C., JONES, M. A. & TOTEMEYER, S. 2018. Novel inflammatory cell infiltration scoring system to investigate healthy and footrot affected ovine interdigital skin. *Peerj,* 6.

ANGELL, J. W., GROVE-WHITE, D. H. & DUNCAN, J. S. 2018. Sheep and farm level factors associated with footrot: a longitudinal repeated cross-sectional study of sheep on six farms in the UK. *Veterinary Record,* 182.

ARGIMON, S., ABUDAHAB, K., GOATER, R. J. E., FEDOSEJEV, A., BHAI, J., GLASNER, C., FEIL, E. J., HOLDEN, M. T. G., YEATS, C. A., GRUNDMANN, H., SPRATT, B. G. & AANENSEN, D. M. 2016. Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. *Microbial Genomics,* 2**,** 11.

ATIA, J., MONAGHAN, E., KALER, J., PURDY, K., GREEN, L. & KEELING, M. 2017. Mathematical modeling of ovine footrot in the UK: the effect of Dichelobacter nodosus and Fusobacterium necrophorum on the disease dynamics. *Epidemics,* 21**,** 13-20.

BATES, D., MACHLER, M., BOLKER, B. M. & WALKER, S. C. 2015. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software,* 67**,** 1-48.

BELLOY, L., GIACOMETTI, M., BOUJON, P. & WALDVOGEL, A. 2007. Detection of Dichelobacter nodosus in wild ungulates (Capra ibex ibex and Ovis aries musimon) and domestic sheep suffering from foot rot using a two-step polymerase chain reaction. *Journal of Wildlife Diseases,* 43**,** 82-88.

BEVERIDGE, W. I. B. 1941. Foot-rot in sheep: A transmissible Disease due to Infection with Fusiformis nodosus (n. sp.). *Council for Scientific and Industrial Research, Bulletin 140***,** 1-56.

BHAT, M. A., WANI, S. A., MUZAFAR, M., RATHER, M. A., TAKU, A. K. & KHANDEY, F. 2013. Non-specificity of primers used for PCR based serogrouping of Dichelobacter nodosus and identification of a novel D. nodosus strain. *Anaerobe,* 21**,** 58-61.

BLANCHARD, A. M., JOLLEY, K. A., MAIDEN, M. C. J., COFFEY, T. J., MABONI, G., STALEY, C. E., BOLLARD, N. J., WARRY, A., EMES, R. D., DAVIES, P. L. & TOTEMEYER, S. 2018. The Applied Development of a Tiered Multilocus Sequence Typing (MLST) Scheme for Dichelobacter nodosus. *Frontiers in Microbiology,* 9.

BULLER, N. B., ASHLEY, P., PALMER, M., PITMAN, D., RICHARDS, R. B. & HAMPSON, D. J. 2010. Understanding the Molecular Epidemiology of the Footrot Pathogen Dichelobacter nodosus To Support Control and Eradication Programs. *Journal of Clinical Microbiology,* 48**,** 877-882.

CAGATAY, I. T. & HICKFORD, J. G. H. 2005. Update on ovine footrot in New Zealand: Isolation, identification, and characterization of Dichelobacter nodosus trains. *Veterinary Microbiology,* 111**,** 171-180.

CAGATAY, T. I. & HICKFORD, J. 2011. Serotyping Dichelobacter nodosus with PCR-SSCP. *Journal of Animal and Veterinary Advances,* 10**,** 1678-1682.

CALVO-BADO, L. A., GREEN, L. E., MEDLEY, G. F., UL-HASSAN, A., GROGONO-THOMAS, R., BULLER, N., KALER, J., RUSSELL, C. L., KENNAN, R. M., ROOD, J. I. & WELLINGTON, E. M. H. 2011a. Detection and diversity of a putative novel heterogeneous polymorphic proline-glycine repeat (Pgr) protein in the footrot pathogen Dichelobacter nodosus. *Veterinary Microbiology,* 147**,** 358-366.

CALVO-BADO, L. A., OAKLEY, B. B., DOWD, S. E., GREEN, L. E., MEDLEY, G. F., UL-HASSAN, A., BATEMAN, V., GAZE, W., WITCOMB, L., GROGONO-THOMAS, R., KALER, J., RUSSELL, C. L. & WELLINGTON, E. M. H. 2011b. Ovine pedomics: the first study of the ovine foot 16S rRNA-based microbiome. *Isme Journal,* 5**,** 1426-1437.

CEDERLOF, S. E., HANSEN, T., KLAAS, I. C. & ANGEN, O. 2013. An evaluation of the ability of Dichelobacter nodosus to survive in soil. *Acta Veterinaria Scandinavica,* 55.

CHEETHAM, B. F., TANJUNG, L. R., SUTHERLAND, M., DRUITT, J., GREEN, G., MCFARLANE, J., BAILEY, G. D., SEAMAN, J. T. & KATZ, M. E. 2006. Improved diagnosis of virulent ovine footrot using the intA gene. *Veterinary Microbiology,* 116**,** 166-174.

CLAXTON, P. D., RIBEIRO, L. A. & EGERTON, J. R. 1983. Classification of Bacteroides-Nodosus by Agglutination Tests. *Australian Veterinary Journal,* 60**,** 331-334.
CLIFTON, R., GIEBE, K., LIU, N., PURDY, K. J. & GREEN, L. E. 2019. Sites of persistence of Fusobacterium necrophorum and Dichelobacter nodosus: a paradigm shift in understanding the epidemiology of footrot in sheep. *Scientific Reports,* 9**,** 11.
CROUCHER, N. J., PAGE, A. J., CONNOR, T. R., DELANEY, A. J., KEANE, J. A., BENTLEY, S. D., PARKHILL, J. & HARRIS, S. R. 2015. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Research,* 43**,** 13.
DE CARVALHO, V. S., SCHERER, C. F. C., LENTS, M. P., GUIMARAES, J. E., MACEDO, J., NASCIMENTO, K. A. & PEDROSO, P. M. O. 2018. Isolation and Molecular Characterization of Dichelobacter nodosus Isolated from Sheep in Bahia, Brazil. *Acta Scientiae Veterinariae,* 46.
DEPIAZZI, L. J., RICHARDS, R. B., HENDERSON, J., ROOD, J. I., PALMER, M. & PENHALE, W. J. 1991. Characterization of Virulent and Benign Strains of Bacteroides-Nodosus. *Veterinary Microbiology,* 26**,** 151-160.
DETTMAN, J. R., RODRIGUE, N. & KASSEN, R. 2015. Genome-Wide Patterns of Recombination in the Opportunistic Human Pathogen Pseudomonas aeruginosa. *Genome Biology and Evolution,* 7**,** 18-34.
DHUNGYEL, O., HUNTER, J. & WHITTINGTON, R. 2014. Footrot vaccines and vaccination. *Vaccine,* 32**,** 3139-3146.
DHUNGYEL, O., SCHILLER, N., EPPLESTON, J., LEHMANN, D., NILON, P., EWERS, A. & WHITTINGTON, R. 2013a. Outbreak-specific monovalent/bivalent vaccination to control and eradicate virulent ovine footrot. *Vaccine,* 31**,** 1701-1706.
DHUNGYEL, O., SCHILLER, N. & WHITTINGTON, R. 2015. Identification and characterization of serogroup M Dichelobacter nodosus from sheep with virulent footrot. *Veterinary Microbiology,* 176**,** 378-381.
DHUNGYEL, O. P., HILL, A. E., DHAND, N. K. & WHITTINGTON, R. J. 2013b. Comparative study of the commonly used virulence tests for laboratory diagnosis of ovine footrot caused by Dichelobacter nodosus in Australia. *Veterinary Microbiology,* 162**,** 756-760.
DHUNGYEL, O. P., WHITTINGTON, R. J. & EGERTON, J. R. 2002. Serogroup specific single and multiplex PCR with pre-enrichment culture and immuno-magnetic bead capture for identifying strains of D-nodosus in sheep with footrot prior to vaccination. *Molecular and Cellular Probes,* 16**,** 285-296.
DIDELOT, X., CROUCHER, N. J., BENTLEY, S. D., HARRIS, S. R. & WILSON, D. J. 2018. Bayesian inference of ancestral dates on bacterial phylogenetic trees. *Nucleic Acids Research,* 46**,** 11.
DIDELOT, X., MERIC, G., FALUSH, D. & DARLING, A. E. 2012. Impact of homologous and non-homologous recombination in the genomic evolution of Escherichia coli. *Bmc Genomics,* 13**,** 15.
DUNCAN, J. S., GROVE-WHITE, D., MOKS, E., CARROLL, D., OULTRAM, J. W., PHYTHIAN, C. J. & WILLIAMS, H. W. 2012. Impact of footrot vaccination and antibiotic therapy on footrot and contagious ovine digital dermatitis. *Veterinary Record,* 170**,** 462-U61.
EGERTON, J. R. & ROBERTS, D. S. 1969. The aetiology and pathogenesis of ovine foot-rot. *Journal of Comparative Pathology,* 79**,** 207-217.
EGERTON, J. R. & ROBERTS, D. S. 1971. VACCINATION AGAINST OVINE FOOT-ROT. *Journal of Comparative Pathology,* 81**,** 179-&.
FODDAI, A., GREEN, L. E., MASON, S. A. & KALER, J. 2012. Evaluating observer agreement of scoring systems for foot integrity and footrot lesions in sheep. *Bmc Veterinary Research,* 8**,** 8.
FROSTH, S., KONIG, U., NYMAN, A. K. & ASPAN, A. 2017. Sample pooling for real-time PCR detection and virulence determination of the footrot pathogen Dichelobacter nodosus. *Veterinary Research Communications,* 41**,** 189-193.
FROSTH, S., KONIG, U., NYMAN, A. K., PRINGLE, M. & ASPAN, A. 2015. Characterisation of Dichelobacter nodosus and detection of Fusobacterium necrophorum and Treponema spp.

in sheep with different clinical manifestations of footrot. *Veterinary Microbiology,* 179**,** 82-90.
FROSTH, S., SLETTEMEAS, J. S., JORGENSEN, H. J., ANGEN, O. & ASPAN, A. 2012. Development and comparison of a real-time PCR assay for detection of Dichelobacter nodosus with culturing and conventional PCR: harmonisation between three laboratories. *Acta Veterinaria Scandinavica,* 54.
GHIMIRE, S. C., EGERTON, J. R. & DHUNGYEL, O. P. 1996. Characterisation of Dichelobacter nodosus isolated from footrot in sheep and goats in Nepal. *Small Ruminant Research,* 23**,** 59-67.
GHIMIRE, S. C., EGERTON, J. R., DHUNGYEL, O. P. & JOSHI, H. D. 1998. Identification and characterisation of serogroup M among Nepalese isolates of Dichelobacter nodosus, the transmitting agent of footrot in small ruminants. *Veterinary Microbiology,* 62**,** 217-233.
GILHUUS, M., KVITLE, B., L'ABEE-LUND, T. M., VATN, S. & JORGENSEN, H. J. 2014. A recently introduced Dichelobacter nodosus strain caused an outbreak of footrot in Norway. *Acta Veterinaria Scandinavica,* 56.
GILHUUS, M., VATN, S., DHUNGYEL, O. P., TESFAMICHAEL, B., L'ABEE-LUND, T. M. & JORGENSEN, H. J. 2013. Characterisation of Dichelobacter nodosus isolates from Norway. *Veterinary Microbiology,* 163**,** 142-148.
GRADIN, J. L., SONN, A. E. & PETROVSKA, L. 1993. Serogrouping of Bacteroides-Nodosus Isolates from 62 Sources in the United-States. *American Journal of Veterinary Research,* 54**,** 1069-1073.
GRAHAM, N. P. H. & EGERTON, J. R. 1968. Pathogenesis of ovine foot-rot: The role of some environmental factors. *Australian Veterinary Journal,* 44**,** 235-240.
GREBER, D., LOCHER, I., KUHNERT, P., BUTTY, M. A., HOLDENER, K., FREY, J., SCHUPBACH-REGULA, G. & STEINER, A. 2018. Pooling of interdigital swab samples for PCR detection of virulent Dichelobacter nodosus. *Journal of Veterinary Diagnostic Investigation,* 30**,** 205-210.
GREEN, L. E. & GEORGE, T. R. N. 2008. Assessment of current knowledge of footrot in sheep with particular reference to Dichelobacter nodosus and implications for elimination or control strategies for sheep in Great Britain. *Veterinary Journal,* 175**,** 173-180.
GREEN, L. E., WASSINK, G. J., GROGONO-THOMAS, R., MOORE, L. J. & MEDLEY, G. F. 2007. Looking after the individual to reduce disease in the flock: A binomial mixed effects model investigating the impact of individual sheep management of footrot and interdigital dermatitis in a prospective longitudinal study on one farm. *Preventive Veterinary Medicine,* 78**,** 172-178.
HADFIELD, J., CROUCHER, N. J., GOATER, R. J., ABUDAHAB, K., AANENSEN, D. M. & HARRIS, S. R. 2018. Phandango: an interactive viewer for bacterial population genomics. *Bioinformatics,* 34**,** 292-293.
HAN, X. Y., KENNAN, R. M., DAVIES, J. K., REDDACLIFF, L. A., DHUNGYEL, O. P., WHITTINGTON, R. J., TURNBULL, L., WHITCHURCH, C. B. & ROOD, J. I. 2008. Twitching motility is essential for virulence in Dichelobacter nodosus. *Journal of Bacteriology,* 190**,** 3323-3335.
HOBBS, M., DALRYMPLE, B. P., COX, P. T., LIVINGSTONE, S. P., DELANEY, S. F. & MATTICK, J. S. 1991. Organization of the Fimbrial Gene Region of Bacteroides-Nodosus - Class-I and Class-Ii Strains. *Molecular Microbiology,* 5**,** 543-560.
HUNT, J. D., JACKSON, D. C., WOOD, P. R., STEWART, D. J. & BROWN, L. E. 1995. Immunological parameters associated with antigenic competition in a multivalent footrot vaccine. *Vaccine,* 13**,** 1649-1657.
JOHN, G. H., SMITH, R., ABRAHAM, K. J. & ELLIS, R. P. 1999. Identification and grouping of Dichelobacter nodosus, using PCR and sequence analysis. *Molecular and Cellular Probes,* 13**,** 61-65.
KALER, J. 2008. *Epidemiological investigations into lameness in sheep.* PhD thesis, University of Warwick.

KALER, J., DANIELS, S. L. S., WRIGHT, J. L. & GREEN, L. E. 2010a. Randomized Clinical Trial of Long-Acting Oxytetracycline, Foot Trimming, and Flunixine Meglumine on Time to Recovery in Sheep with Footrot. *Journal of Veterinary Internal Medicine,* 24**,** 420-425.
KALER, J. & GREEN, L. E. 2008. Naming and recognition of six foot lesions of sheep using written and pictorial information: A study of 809 English sheep farmers. *Preventive Veterinary Medicine,* 83**,** 52-64.
KALER, J. & GREEN, L. E. 2009. Farmers' practices and factors associated with the prevalence of all lameness and lameness attributed to interdigital dermatitis and footrot in sheep flocks in England in 2004. *Preventive Veterinary Medicine,* 92**,** 52-59.
KALER, J., MEDLEY, G. F., GROGONO-THOMAS, R., WELLINGTON, E. M. H., CALVO-BADO, L. A., WASSINK, G. J., KING, E. M., MOORE, L. J., RUSSELL, C. & GREEN, L. E. 2010b. Factors associated with changes of state of foot conformation and lameness in a flock of sheep. *Preventive Veterinary Medicine,* 97**,** 237-244.
KALER, J., WASSINK, G. J. & GREEN, L. E. 2009. The inter- and intra-observer reliability of a locomotion scoring scale for sheep. *Veterinary Journal,* 180**,** 189-194.
KENNAN, R. M., DHUNGYEL, O. P., WHITTINGTON, R. J., EGERTON, J. R. & ROOD, J. I. 2003. Transformation-mediated serogroup conversion of Dichelobacter nodosus. *Veterinary Microbiology,* 92**,** 169-178.
KENNAN, R. M., GILHUUS, M., FROSTH, S., SEEMANN, T., DHUNGYEL, O. P., WHITTINGTON, R. J., BOYCE, J. D., POWELL, D. R., ASPAN, A., JORGENSEN, H. J., BULACH, D. M. & ROOD, J. I. 2014. Genomic Evidence for a Globally Distributed, Bimodal Population in the Ovine Footrot Pathogen Dichelobacter nodosus. *Mbio,* 5.
KENNAN, R. M., HAN, X. Y., PORTER, C. J. & ROOD, J. I. 2011. The pathogenesis of ovine footrot. *Veterinary Microbiology,* 153**,** 59-66.
KENNAN, R. M., LOVITT, C. J., HAN, X. Y., PARKER, D., TURNBULL, L., WHITCHURCH, C. B. & ROOD, J. I. 2015. A two-component regulatory system modulates twitching motility in Dichelobacter nodosus. *Veterinary Microbiology,* 179**,** 34-41.
KENNAN, R. M., WONG, W., DHUNGYEL, O. P., HAN, X. Y., WONG, D., PARKER, D., ROSADO, C. J., LAW, R. H. P., MCGOWAN, S., REEVE, S. B., LEVINA, V., POWERS, G. A., PIKE, R. N., BOTTOMLEY, S. P., SMITH, A. I., MARSH, I., WHITTINGTON, R. J., WHISSTOCK, J. C., PORTER, C. J. & ROOD, J. I. 2010. The Subtilisin-Like Protease AprV2 Is Required for Virulence and Uses a Novel Disulphide-Tethered Exosite to Bind Substrates. *Plos Pathogens,* 6.
KEOGH, J. 1976. Footrot vaccine trials. *New South Wales Veterinary Proceedings,* 12**,** 35-37.
KNAPPE-POINDECKER, M., GILHUUS, M., JENSEN, T. K., VATN, S., JORGENSEN, H. J. & FJELDAAS, T. 2014. Cross-infection of virulent Dichelobacter nodosus between sheep and co-grazing cattle. *Veterinary Microbiology,* 170**,** 375-382.
KUMAR, N. V., SREENIVASULU, D. & KARTHIK, A. 2016. Identification and characterization of Dichelobacter nodosus serogroup H from ovine footrot in India. *Anaerobe,* 40**,** 100-102.
LAFONTAINE, S., EGERTON, J. R. & ROOD, J. I. 1993. Detection of Dichelobacter-Nodosus Using Species-Specific Oligonucleotides as Pcr Primers. *Veterinary Microbiology,* 35**,** 101-117.
LEY, S. J., WATERMAN, A. E. & LIVINGSTON, A. 1995. A FIELD-STUDY OF THE EFFECT OF LAMENESS ON MECHANICAL NOCICEPTIVE THRESHOLDS IN SHEEP. *Veterinary Record,* 137**,** 85-87.
LI, H., HANDSAKER, B., WYSOKER, A., FENNELL, T., RUAN, J., HOMER, N., MARTH, G., ABECASIS, G., DURBIN, R. & GENOME PROJECT DATA, P. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics,* 25**,** 2078-2079.
LOCHER, I., GIGER, L., FROSTH, S., KUHNERT, P. & STEINER, A. 2018. Potential transmission routes of Dichelobacter nodosus. *Veterinary Microbiology,* 218**,** 20-24.
LOCHER, I., GREBER, D., HOLDENER, K., LUCHINGER, R., HAERDI-LANDERER, C., SCHUPBACH-REGULA, G., FREY, J. & STEINER, A. 2015. Longitudinal Dichelobacter nodosus status in 9 sheep flocks free from clinical footrot. *Small Ruminant Research,* 132**,** 128-132.

MABONI, G., DAVENPORT, R., SESSFORD, K., BAIKER, K., JENSEN, T. K., BLANCHARD, A. M., WATTEGEDERA, S., ENTRICAN, G. & TOTEMEYER, S. 2017. A Novel 3D Skin Explant Model to Study Anaerobic Bacterial Infection. *Frontiers in Cellular and Infection Microbiology,* 7.

MATTICK, J. S., ANDERSON, B. J., COX, P. T., DALRYMPLE, B. P., BILLS, M. M., HOBBS, M. & EGERTON, J. R. 1991. GENE-SEQUENCES AND COMPARISON OF THE FIMBRIAL SUBUNITS REPRESENTATIVE OF BACTEROIDES-NODOSUS SEROTYPE-A TO SEROTYPE-I - CLASS-I AND CLASS-II STRAINS. *Molecular Microbiology,* 5**,** 561-573.

MCPHERSON, A. S., DHUNGYEL, O. P. & WHITTINGTON, R. J. 2017. Evaluation of Genotypic and Phenotypic Protease Virulence Tests for Dichelobacter nodosus Infection in Sheep. *Journal of Clinical Microbiology,* 55**,** 1313-1326.

MCPHERSON, A. S., DHUNGYEL, O. P. & WHITTINGTON, R. J. 2018. Detection and Serogrouping of Dichelobacter nodosus Infection by Use of Direct PCR from Lesion Swabs To Support Outbreak-Specific Vaccination for Virulent Footrot in Sheep. *Journal of Clinical Microbiology,* 56.

MELING, S. & ULVUND, M. J. 2009. Flock health visits in 17 sheep flocks in Rogaland. *Stuen, S., Ulvuind, M. J. (Eds.) Proceedings of the 7th International Sheep Veterinary Congress, Stavanger, Norway***,** 148-149.

MOORE, L. J., WASSINK, G. J., GREEN, L. E. & GROGONO-THOMAS, R. 2005. The detection and characterisation of Dichelobacter nodosus from cases of ovine footrot in England and Wales. *Veterinary Microbiology,* 108**,** 57-67.

MOSES, E. K., GOOD, R. T., SINISTAJ, M., BILLINGTON, S. J., LANGFORD, C. J. & ROOD, J. I. 1995. A MULTIPLE SITE-SPECIFIC DNA-INVERSION MODEL FOR THE CONTROL OF OMP1 PHASE AND ANTIGENIC VARIATION IN DICHELOBACTER-NODOSUS. *Molecular Microbiology,* 17**,** 183-196.

MUZAFAR, M., GREEN, L. E., CALVO-BADO, L. A., TICHAUER, E., KING, H., JAMES, P. & WELLINGTON, E. M. H. 2016. Survival of the ovine footrot pathogen Dichelobacter nodosus in different soils. *Anaerobe,* 38**,** 81-87.

MYERS, G. S. A., PARKER, D., AL-HASANI, K., KENNAN, R. M., SEEMANN, T., REN, Q. H., BADGER, J. H., SELENGUT, J. D., DEBOY, R. T., TETTELIN, H., BOYCE, J. D., MCCARL, V. P., HAN, X. Y., NELSON, W. C., MADUPU, R., MOHAMOUD, Y., HOLLEY, T., FEDOROVA, N., KHOURI, H., BOTTOMLEY, S. P., WHITTINGTON, R. J., ADLER, B., SONGER, J. G., ROOD, J. I. & PAULSEN, I. T. 2007. Genome sequence and identification of candidate vaccine antigens from the animal pathogen Dichelobacter nodosus. *Nature Biotechnology,* 25**,** 569-575.

NIEUWHOF, G. J. & BISHOP, S. C. 2005. Costs of the major endemic diseases of sheep in Great Britain and the potential benefits of reduction in disease impact. *Animal Science,* 81**,** 23-29.

O'LEARY, N. A. W., M. W.; BRISTER, R.; CIUFO, S.; HADDAD, D.; MCVEIGH, R.; RAJPUT, B.; ROBBERTSE, B.; SMITH-WHITE, B; AKO-ADJEI, D.; ASTASHYN, A.; BADRETDIN, A.; BAO, Y.; BLINKOVA, O.; BROVER, V.; CHETVERNIN, V.; CHOI, J.; COX, E.; ERMOLAEVA, O.; FARRELL, C. M.; GOLDFARB, T.; GUPTA, T.; HAFT, D.; HATCHER, E.; HLAVINA, W.; JOARDAR, V. S.; KODALI, V. K.; LI, W.; MAGLOTT, D.; MASTERSON, P.; MCGARVEY, K. M.; MURPHY, M. R.; O'NEILL, K.; PUJAR, S.; RANGWALA, S. H.; RAUSCH, D.; RIDDICK, L. D.; SCHOCH, C.; SHKEDA, A.; STORZ, S. S.; SUN, H.; THIBAUD-NISSEN, F.; TOLSTOY, I.; TULLY, R. E.; VATSAN, A. R.; WALLIN, C.; WEBB, D.; WU, W.; LANDRUM, M. J.; KIMCHI, A.; TATUSOVA, T.; DICUCCIO, M.; KITTS, P.; MURPHY, T. D. & PRUITT, K. D. 2016. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. *Nucleic Acids Research,* 44**,** D733-D745.

ONDOV, B. D., TREANGEN, T. J., MELSTED, P., MALLONEE, A. B., BERGMAN, N. H., KOREN, S. & PHILLIPPY, A. M. 2016. Mash: fast genome and metagenome distance estimation using MinHash. *Genome Biology,* 17**,** 14.

OSOVA, A., PILIPCINCOVA, I. S. B., KIRALY, J., DOLNIK, M. & MUDRON, P. 2018. Assessment of two different methods for sampling and detection of Dichelobacter nodosus and

Fusobacterium necrophorum in dairy cows in Eastern Slovakia. *Journal of Applied Animal Research,* 46**,** 1452-1456.
PAGE, A. J., DE SILVA, N., HUNT, M., QUAIL, M. A., PARKHILL, J., HARRIS, S. R., OTTO, T. D. & KEANE, J. A. 2016. Robust high-throughput prokaryote de novo assembly and improvement pipeline for Illumina data. *Microbial Genomics,* 2**,** 7.
PALMER, M. A. 1993. A Gelatin Test to Detect Activity and Stability of Proteases Produced by Dichelobacter (Bacteroides) Nodosus. *Veterinary Microbiology,* 36**,** 113-122.
RAMBAUT, A., LAM, T. T., CARVALHO, L. M. & PYBUS, O. G. 2016. Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). *Virus Evolution,* 2**,** 7.
RIFFKIN, M. C., WANG, L. F., KORTT, A. A. & STEWART, D. J. 1995. A single amino-acid change between the antigenically different extracellular serine proteases V2 and B2 from Dichelobacter nodosus. *Gene,* 167**,** 279-283.
ROBERTS, D. S. & EGERTON, J. R. 1969. The Aetiology and Pathogenesis of Ovine Foot-rot II. The Pathogenic Association of Fusiformis nodosus and F. necrophorus. *Journal of Comparative Pathology,* 79**,** 217-227.
ROGDO, T., HEKTOEN, L., SLETTEMEAS, J. S., JORGENSEN, H. J., OSTERAS, O. & FJELDAAS, T. 2012. Possible cross-infection of Dichelobacter nodosus between co-grazing sheep and cattle. *Acta Veterinaria Scandinavica,* 54.
ROOD, J. I., HOWARTH, P. A., HARING, V., BILLINGTON, S. J., YONG, W. K., LIU, D., PALMER, M. A., PITMAN, D. R., LINKS, I., STEWART, D. J. & VAUGHAN, J. A. 1996. Comparison of gene probe and conventional methods for the differentiation of ovine footrot isolates of Dichelobacter nodosus. *Veterinary Microbiology,* 52**,** 127-141.
RUSSELL, C. L., SMITH, E. M., CALVO-BADO, L. A., GREEN, L. E., WELLINGTON, E. M. H., MEDLEY, G. F., MOORE, L. J. & GROGONO-THOMAS, R. 2014. Multiple locus VNTR analysis highlights that geographical clustering and distribution of Dichelobacter nodosus, the causal agent of footrot in sheep, correlates with inter-country movements. *Infection Genetics and Evolution,* 22**,** 273-279.
SCHWARTZKOFF, C. L., EGERTON, J. R., STEWART, D. J., LEHRBACH, P. R., ELLEMAN, T. C. & HOYNE, P. A. 1993. The Effects of Antigenic-Competition on the Efficacy of Multivalent Footrot Vaccines. *Australian Veterinary Journal,* 70**,** 123-126.
SMITH, E. M., GILBERT, A., RUSSELL, C. L., PURDY, K. J., MEDLEY, G. F., MUZAFAR, M., GROGONO-THOMAS, R. & GREEN, L. E. 2017. Within-Flock Population Dynamics of *Dichelobacter nodosus*. *Front Vet Sci,* 4**,** 1-6.
SMITH, E. M., GREEN, O. D. J., CALVO-BADO, L. A., WITCOMB, L. A., GROGONO-THOMAS, R., RUSSELL, C. L., BROWN, J. C., MEDLEY, G. F., KILBRIDE, A. L., WELLINGTON, E. M. H. & GREEN, L. E. 2014. Dynamics and impact of footrot and climate on hoof horn length in 50 ewes from one farm over a period of 10 months. *Veterinary Journal,* 201**,** 295-301.
STAMATAKIS, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics,* 30**,** 1312-1313.
STÄUBLE, A., STEINER, A., FREY, J. & KUHNERT, P. 2014. Simultaneous Detection and Discrimination of Virulent and Benign Dichelobacter nodosus in Sheep of Flocks Affected by Foot Rot and in Clinically Healthy Flocks by Competitive Real-Time PCR. *Journal of Clinical Microbiology,* 52**,** 1228-1231.
STÄUBLE, A., STEINER, A., NORMAND, L., KUHNERT, P. & FREY, J. 2014. Molecular genetic analysis of Dichelobacter nodosus proteases AprV2/B2, AprV5/B5 and BprV/B in clinical material from European sheep flocks. *Veterinary Microbiology,* 168**,** 177-184.
STEWART, D. J. 1979. Role of Elastase in the Differentiation of Bacteroides-Nodosus Infections in Sheep and Cattle. *Research in Veterinary Science,* 27**,** 99-105.
THORLEY, C. M. D., S. E. J. 1986. Serotyping survey of 1296 strains of *Bacteroides nodosus* isolated fro sheep and cattle in Great Britain and western Europe. *Footrot in ruminants: proceedings of a workshop, Melbourne 1985***,** 135-142.

WANI, A. H., VERMA, S., SHARMA, M. & WANI, S. A. 2015. Infectious lameness among migratory sheep and goats in north-west India, with particular focus on anaerobes. *Revue Scientifique Et Technique-Office International Des Epizooties,* 34**,** 855-867.
WASSINK, G. J., GROGONO-THOMAS, R., MOORE, L. J. & GREEN, L. E. 2003. Risk factors associated with the prevalence of footrot in sheep from 1999 to 2000. *Veterinary Record,* 152**,** 351-358.
WASSINK, G. J., GROGONO-THOMAS, R., MOORE, L. J. & GREEN, L. E. 2004. Risk factors associated with the prevalence of interdigital dermatitis in sheep from 1999 to 2000. *Veterinary Record,* 154**,** 551-+.
WASSINK, G. J., KING, E. M., GROGONO-THOMAS, R., BROWN, J. C., MOORE, L. J. & GREEN, L. E. 2010. A within farm clinical trial to compare two treatments (parenteral antibacterials and hoof trimming) for sheep lame with footrot. *Preventive Veterinary Medicine,* 96**,** 93-103.
WINTER, A. C. 2008. Lameness in sheep. *Small Ruminant Research,* 76**,** 149-153.
WINTER, J. R., KALER, J., FERGUSON, E., KILBRIDE, A. L. & GREEN, L. E. 2015. Changes in prevalence of, and risk factors of, lameness in random samples of English sheep flocks: 2004-2013. *Preventative Veterinary Medicine*.
WITCOMB, L. A., GREEN, L. E., CALVO-BADO, L. A., RUSSELL, C. L., SMITH, E. M., GROGONO-THOMAS, R. & WELLINGTON, E. M. H. 2015. First study of pathogen load and localisation of ovine footrot using fluorescence in situ hybridisation (FISH). *Veterinary Microbiology,* 176**,** 321-327.
WITCOMB, L. A., GREEN, L. E., KALER, J., UL-HASSAN, A., CALVO-BADO, L. A., MEDLEY, G. F., GROGONO-THOMAS, R. & WELLINGTON, E. M. H. 2014. A longitudinal study of the role of Dichelobacter nodosus and Fusobacterium necrophorum load in initiation and severity of footrot in sheep. *Preventive Veterinary Medicine,* 115**,** 48-55.
ZINICOLA, M., HIGGINS, H., LIMA, S., MACHADO, V., GUARD, C. & BICALHO, R. 2015. Shotgun Metagenomic Sequencing Reveals Functional Genes and Microbiome Associated with Bovine Digital Dermatitis. *Plos One,* 10**,** 17.

8 Appendices

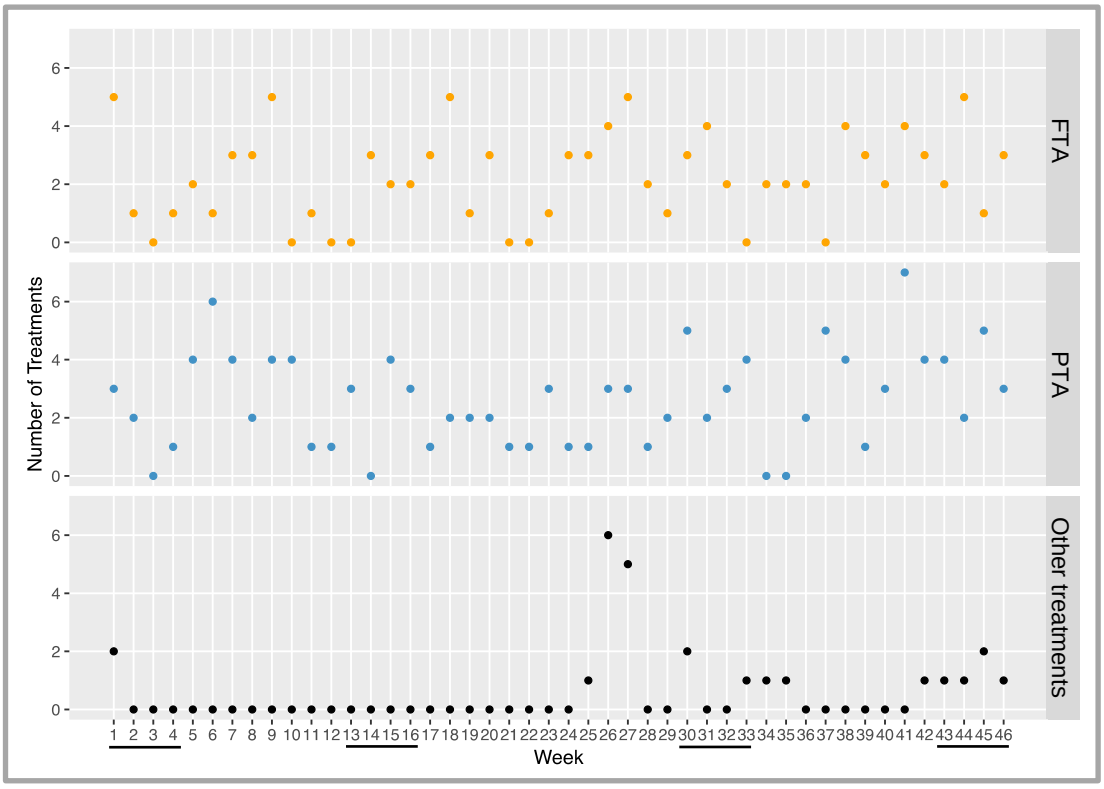
On the following pages.

Appendix 1: Distribution of the 925 foot swabs by extractor.

	EXTRACTED 2010/2011 EXTRACTOR 1		EXTRACTED 2010/2011 EXTRACTOR 2		EXTRACTED 2016 EXTRACTOR 3			
Sheep ID	**Interdigital skin samples**	**Non-interdigital lesion samples**	**Interdigital skin samples**	**Non-interdigital lesion samples**	**Interdigital skin samples**	**Non-interdigital lesion samples**	**Missing**	**Removed**
0076			36	2	2		2	1[a]
0077					48	7		
0078					26			
0081			24	4	2		4	
0084			2		28	2		
0088			26	3	2		5	
0089			30		2	2	2	
0092					32	1		
0093	16				16	3		
0094					32	2		
0095	14				12			
0096	18	1			16	1		
0101					30	5		
0102					34	3		
0103			26	5	2		4	
0104			34		2			
0106			2		30	1		
0107					35	5		1[b]
0111			2		38	5	2	
0112			2		50	6		
0113			2		50	9		
0117			2		36	2		
0119			2		26	4		
0121			2		26	3		
0124	16	2			12			
Total	**64**	**3**	**192**	**14**	**589**	**61**	**19**	**1**
	923							

[a] Two swabs were taken for RF 21/10/10 – therefore have calculated an average *D. nodosus* load of both resulting in 36 samples.

[b] Mix up between LR and RF samples from 28/01/2011 so cannot use selected LR sample.



Appendix 2: Number of treatments given each week for the 91 ewes from the Bristol study flock.

Treatments labelled on the righthand side. **FTA** – foot trim plus topical antibiotic foot spray. **PTA** – parenteral antibiotics plus topical antibiotic foot spray, weeks: routine sampling weeks.

Appendix 3: Results from each separate fixed effect used in the multivariable binomial mixed effects regression model of *D. nodosus* detection.

	D. nodosus DETECTION			
Predictors	**Odds R.**	**CI**	**CS**	**p value**
Foot region (Interdigital skin)	1.94	0.97 – 3.88	1.86	0.063
Routine or Targeted (Targeted)	0.65	0.22 – 1.87	-0.81	0.420
Locomotion score 1	2.39	0.40 – 14.42	0.95	0.341
Locomotion score 2	0.59	0.23 – 1.55	-1.07	0.285
Locomotion score 3	3.17	1.74 – 5.79	3.76	**< 0.001**
Locomotion score 4**	-	-	-	-
ID score 1	1.47	0.89 – 2.43	1.50	0.134
ID score 2	6.65	2.53 – 17.49	3.84	**< 0.001**
ID score 3	10.75	2.78 – 41.59	3.44	**0.001**
ID score 4	14.40	4.33 – 47.91	4.35	**< 0.001**
SFR score 1	0.91	0.58 – 1.43	-0.41	0.679
Culture positive**	-	-	-	-
References:				
Foot region (Non-interdigital lesion)				
Routine or Targeted (Routine)				
Locomotion score 0				
ID score 0				
SFR score 0				
Culture negative				

CI: Confidence intervals.
CS: Coefficient statistic.
Odds R.: Odds Ratios.
**All samples were *D. nodosus* positive or only one sample was negative.

Appendix 4: Results from each separate fixed effect used in the multivariable linear mixed effects regression model of log_{10}(*D. nodosus* load +1).

	LOG$_{10}$(*D. nodosus* LOAD +1)		
Predictors	**Estimates**	**CI**	**p value**
Foot region* (Interdigital skin)	1.17	0.72 – 1.62	**< 0.001**
Routine or Targeted (Targeted)	-0.66	-1.34 – -0.01	0.055
Locomotion score 1	0.74	-0.57 – 2.06	0.269
Locomotion score 2	-0.22	-0.92 – 0.47	0.529
Locomotion score 3	0.97	0.57 – 1.36	**< 0.001**
Locomotion score 4	2.15	1.20 – 3.11	**< 0.001**
ID score 1	0.46	0.14 – 0.79	**0.005**
ID score 2	1.40	0.96 – 1.85	**< 0.001**
ID score 3	1.64	1.07 – 2.22	**< 0.001**
ID score 4	2.18	1.62 – 2.73	**< 0.001**
SFR score 1	-0.05	-0.35 – 0.26	0.769
Culture positive	1.69	1.16 – 2.22	**< 0.001**
References:			
Foot region (Non-interdigital lesion)			
Routine or Targeted (Routine)			
Locomotion score 0			
ID score 0			
SFR score 0			
Culture negative			

CI: Confidence intervals.

Appendix 5: Results from each separate fixed effect used in the multivariable binomial mixed effects regression model of *D. nodosus* serogroup detection on ID score ≥ 2.

	ID SCORE 2 - 4			
Predictors	**Odds R.**	**CI**	**CS**	**p value**
B positive	1.78	0.75 – 4.25	1.31	0.191
H positive	1.12	0.55 – 2.29	0.32	0.748
I positive	0.98	0.47 – 2.05	-0.06	0.953
D positive	1.15	0.38 – 3.48	0.25	0.805
References:				
B negative				
H negative				
I negative				
D negative				

CI: Confidence intervals.
CS: Coefficient statistic.
Odds R.: Odds Ratios.

Appendix 6: Results from each separate fixed effect used in the multivariable binomial mixed effects regression model of *D. nodosus* serogroup detection on positive SFR score.

	SFR SCORE 1			
Predictors	**Odds R.**	**CI**	**CS**	**p value**
B positive	1.11	0.44 – 2.79	0.21	0.831
H positive	0.73	0.36 – 1.52	-0.83	0.404
I positive	1.75	0.66 – 4.64	1.12	0.261
D positive	0.25	0.04 – 1.43	-1.56	0.119
References:				
B negative				
H negative				
I negative				
D negative				

CI: Confidence intervals.
CS: Coefficient statistic.
Odds R.: Odds Ratios.