Study of the genetic diversity of *Campylobacter lari* from skua species (*Stercorarius maccormicki*, *Catharacta Antarctica*) from the Southern Ocean and Antarctica

Official Master in Zoonosis and One Health

2019-2020

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Acknowledgments

This project becomes a reality with the kind support and help of many individuals. It would be an understatement to say that this year has passed with some ups and downs. Yet, it is through their guidance and encouragement that this paper reached its goal.

Foremost, I wish to express my sincere appreciation to my supervisor, Marta Cerdà, for trusting me with such an amazing opportunity, for your involvement and assessment in each step of the way. I would also like to extend my deepest gratitude to Teresa Ayats, for your patience and for sharing your knowledge and experience, essential in shaping the experiments. Also, to each person that has been involved in the previous stages of this study for their invaluable contributions.

Thanks to everyone at CReSA for their willingness and excellent work environment that made each day lighter. I'm especially grateful to my lab colleagues for your unparalleled support and for teaching me what it's like to be in a great team.

I'd like to acknowledge the assistance and effort of everyone at the Faculty of Veterinary at the UAB for making this project possible even through these challenging times.

Finally, this would not have been possible without the support and nurturing of my family and friends. To my mother, for your loving encouragement, your strength and your ability to always put a smile on my face. To my father for being my rock and the person that better gets me because we have such similar souls. To my brother, for being my best friend, my confident, and for pushing me to be my best self every step of the way. To my grandfather, the light in my sky, for believing in me and knowing my path even before I did. To my grandmother, for being by my side long before I can remember and being an example of bravery and persistence. And to those friends that managed to be by my side despite being an ocean away.

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ABSTRACT

Campylobacteriosis is the most reported gastrointestinal disease in humans in the EU, representing 70% of all the reporting cases. Wild animals, especially birds, are well known reservoirs for *Campylobacter*. The presence of pathogens in the Antarctica has received limited attention due to the region's isolation. However, Antarctic seabirds disperse across the Southern Ocean and can even spend the winter in the northern hemisphere. Such large-scale movements are thought to contribute to the introduction and dispersion of pathogens. To gain insight into de epidemiology and genetic diversity of *Campylobacter*, *C. lari* isolated from south polar skua (*Stercorarius maccormicki)* and brown skua (*Catharacta antarctica)* of the Southern Ocean was analysed. A total of 231 isolates of *C. lari* were recovered and identified from cloacal swab samples of 55 skuas. Birds were sampled at Adelaide Island, Gough Island, Falkland Islands, and Svarthamaren in the Southern Ocean. Restriction fragment length polymorphism (RFLP) was used to characterize the *C. lari* isolates using *fla-A* gene to study the genetic variation. A second typing technique, pulse field gel electrophoresis (PFGE), was used for a higher power resolution in the characterization. Analysis were completed by including RFLP and PFGE data retrieved from a database containing data from *C. lari* isolates from additional locations from the Southern Ocean.

Some profiles were found to be the same in both RFLP and PFGE, which was attributed to the higher discriminatory capacity of PFGE and the high genetic instability of Campylobacter. Through fingerprinting, similarities in pulsotypes between brown and south polar skuas and between sampling sites were found. These similarities can be attributed to the role of migration and hybridization between the skuas. These findings are important for a better understanding of the transmission of pathogens in the Antarctic and its potential impact and relation to human health.

1. Introduction

1.1 Zoonoses

Zoonotic diseases (also known as zoonoses) are defined as any disease or infection transmitted from animals to humans (Chlebicz & Śliżewska, 2018). Zoonoses can be caused by bacterial, viral, fungi, or parasitic agents through (Karesh et al., 2012):

- Direct contact with the infected animal by saliva, blood mucus or other body fluids.
- Indirect contact with areas where the animals live or surfaces that have been contaminated.
- Vector-borne (i.e. mosquitoes, ticks or fleas).
- Food-borne by eating or drinking contaminated food (i.e. raw, unpasteurized, etc).
- Water-borne by drinking or being in contact with contaminated water.

According to the World Health Organization (WHO), 75% of new emerging human infectious diseases are defined as zoonotic (Gebreyes et al., 2014). As for the already known emerging infectious diseases, it is estimated that 60% of the 1,461 infectious diseases recognized to occur in humans by the National Academy of Science, Institute of Medicine, are caused by multi-host pathogens, which means they are shared with wild or domestic animals (Bidaisee & Macpherson, 2014).

Endemic zoonoses are the greatest burden of human health, causing about 1 billion illnesses and millions of deaths every year (Karesh et al., 2012). Enteric zoonotic pathogens are the cause of millions of those illnesses and thousands of deaths worldwide, most cases involve three enteric bacteria -*Campylobacter*, *Salmonella*, and *Escherichia coli*. These cases are usually originated in human, livestock, or wildlife waste (Smith et al., 2020). Among wildlife, birds have been the centre of many studies as they can carry pathogens through large distances because they are very mobile, especially during migration (Fu et al., 2019). Some of the routes through which wild birds can transmit enteric bacteria to humans are direct contact by hunting and consuming the meat, intentional interaction (i.e. feeding), and, more commonly, contact with surfaces contaminated with wild birds' faeces (Smith et al., 2020).

1.1.1 One health approach

One health concept was officially adopted by international organizations in 1984, it is defined by the One Health Initiative Task Force (OITF) as "the promotion, improvement, and defence for the health and well-being of all species by enhancing cooperation and collaboration between physicians, veterinarians, and other scientific health professionals and by promoting strengths in leadership and management to achieve these goals" (Kahn et al., 2016). This multidisciplinary approach has a significant role in the control and prevention of zoonoses since the emergence of infectious diseases is caused by a complex multifactorial set of circumstances. Wildlife represents the most significant threat to global health of all emergence infectious diseases (Jones et al., 2008). Most of these diseases in humans, livestock, and wild animals are thought to be maintained in reservoir hosts. However, the reservoirs are often hard to identify, especially when they are wildlife reservoirs (Haydon et al., 2002). Cross-sectoral collaboration is key to manage the human-animal-environment interface and thus, improve global health (Kelly et al., 2020).

1.2 Campylobacter

The genus *Campylobacter* consists of 47 species and belongs to the family *Campylobacteraceae,* under the order of *Campylobacterales,* in the class of Epsilonproteobacteria and in the phylum of proteobacteria (LPSN, 2020). The family is divided into four genera: *Campylobacter, Arcobacter, Dehalospirilum* and *Sulfurospirilum* (Chlebicz & Śliżewska, 2018).

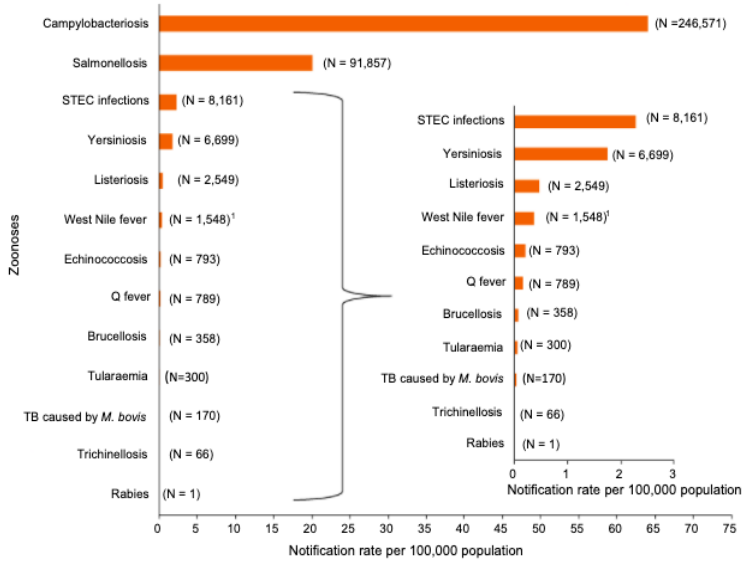
Campylobacter genus are gram-negative bacteria, microaerophilic (survives and grows best in an environment characterized by a low oxygen tension -5% O2, 10% CO2, and 85% N2-) (Facciolà et al., 2017). This genus is non-spore forming and has a length between 0.5 and 5 μm and a width comprised between 0.2 to 0.9 μm (Mardones & López, 2017). The DNA is rich in adenine and thymine and around 1-6-1.7 Mbps. *Campylobacter* species are usually mobile, characterized by a polar flagellum present on one or both ends of the cell that causes a spiral movement (Facciolà et al., 2017). *Campylobacter* are mainly spiral-shaped, "S"-shaped, or curved, rod-shaped bacteria with a typical movement like a corkscrew determined by the helical shaped flagella (Chlebicz & Śliżewska, 2018). The genes responsible for motility in *Campylobacter* species are two flagellin genes *flaA* and *flaB* that can be found in tandem (Cody

et al., 2010). Sequencing of many *Campylobacter* species has been achieved, which has allowed by homologous recombination to determine that *flaA* is necessary for invasion while *flaB* is not expressed as much. These genes undergo intergenic recombination and can further contribute to the bacterial virulence (Meinersmann et al., 2005).

Campylobacter can synthesize the oxidase enzyme, except for *C. gracilis*, and can grow at pH between 6.5 and 7.5 and temperatures between 37-42ºC, being unable to grow at temperatures above 55ºC or below 30ºC. The growth does not occur in environments with water activity (aw) concentrations of less than 0.987 (sensitive to NaCl >2% w/v), while it is optimal if equal to 0.997 (NaCl about 0,5% w / v) (Facciolà et al., 2017). These bacteria are fragile and cannot tolerate drying. Therefore, freezing reduces the number of *Campylobacter* bacteria on raw meat (Chlebicz & Śliżewska, 2018).

1.2.1 Campylobacteriosis

Campylobacteriosis is the most reported gastrointestinal disease in humans in the EU and has been since 2005, representing 70% of all the reported cases, followed by other bacterial diseases such as salmonellosis, STEC (Shiga toxin-producing *E. coli*) infections and yersiniosis (see Figure1). Worldwide, the genus *Campylobacter* is one of four key global causes of diarrhoeal diseases and considered to be the most common cause of bacterial human gastroenteritis (EFSA, 2019; Pérez-Boto et al., 2014). *Campylobacter spp* is found in the gastrointestinal tract of a wide range of wild animals (mostly birds and mammals), farm animals (livestock, pigs, poultry), and pets (dogs and cats) (Facciolà et al., 2017).



Note: The total number of confirmed cases is indicated between parentheses at the end of each bar.
†Exception: West Nile fever where the total number of cases was used.

Figure 1. Notification rates of confirmed human zoonoses in the EU, 2018 (EFSA, 2019).

The transmission usually occurs through faecal-oral route, being the main environmental niche the intestinal tract of all avian species. Poultry consumption represents 50-70% of human cases of campylobacteriosis (Facciolà et al., 2017).

Campylobacteriosis symptoms are acute and characterized by abdominal pain, nausea, dehydration, diarrhoea, fever, and fatigue. Treatment is usually contraindicated except for the most severe cases since the illness is typically self-limiting. Complications are rare, but *Campylobacter* infections can be associated with the autoimmune disorders Miller-Fisher syndrome and Guillain-Barré syndrome, which are neurological disorders characterized by paralysis and weakness of limbs, anaemia, possible involvement of respiratory muscles, and sensory loss, reactive arthritis involving knees and ankles, occurring about a month after infection and for as long as 5 years (Chlebicz & Śliżewska, 2018; WHO, 2012).

The high incidence of campylobacteriosis, along with the duration and complication, makes it a high burden from a socio-economic perspective, especially in developing countries in which diarrhoeal infections are very common in children under the age of 2 years and sometimes results in death (Platts-Mills & Kosek, 2014; WHO, 2012).

From the 47 species and 16 subspecies assigned to Campylobacter, the so-called thermophilic species (able to grow at 42ºC but not below 30ºC) are the causal agents of human gastrointestinal disease (campylobacteriosis). *Campylobacter jejuni* is the cause of approximately 90% of campylobacteriosis, followed in order of importance by *C. coli*, *C. upsaliensis* (found in cats and dogs) and *C. lari* (found in seabirds in particular) (Fouts et al., 2005).

Although most *Campylobacter* infections are considered food-borne, associated with the consumption or handling of contaminated meat, sporadic cases in which the source of infection goes undetermined are common (Meinersmann et al., 2005). Birds are an important reservoir, as some species have shown to be asymptomatic, making the detection challenging and posing a bigger threat to human health. In this context, production animals play an important role, as consumption of uncooked meat is considered a risk factor (Moré et al., 2017). However, in order to achieve a One Health approach to this subject, the role of wild animals must be also considered (Smith et al., 2020).

1.3 Reservoirs

Campylobacter spp is widely distributed in nature, found naturally in soil, manure, and aquatic systems. It is often caused by the contamination of poultry, beef, and pork. Bacteria from the *Campylobacter* genus do not proliferate outside the alimentary tract of warm-blooded animals but may survive for several weeks in food products that have been stored at low temperatures (Chlebicz & Śliżewska, 2018; EFSA, 2019; Jurado Tarifa et al., 2016).

The main *Campylobacter* reservoir are domestic and wild birds, but ruminants such as cattle, goats, and sheep, may act also as an important reservoir for *Campylobacter* (Kemp et al., 2005). Birds are considered a natural host of thermophilic *Campylobacter spp* because their body temperature (41-42ºC) seems to be well adapted to the bacteria (Tang et al., 2017). The bacteria colonize mostly the gut (duodenum, jejunum, and intestines) rather than the rumen. In an infected herd, these bacteria are present in up to 80% of animals (20% in sheep) (Facciolà et al., 2017). Besides the meat, milk can also pose a risk of infection. Raw milk can be contaminated during milking or as a result of udder infections. *Campylobacter spp* can also inhabit the alimentary tract of pigs with an incidence of 38-63%, although infections resulting from the consumption of pork are rare, pigs are considered the main reservoir of *C. coli*. Vegetables are also a frequent vector of transmission as a result of direct or indirect contact with livestock faeces, mainly due to cross contamination during handling at the kitchen (Chlebicz & Śliżewska, 2018; EFSA, 2019).

Wild birds are considered natural vertebrate reservoirs of *Campylobacter spp* and thus are frequently mentioned as possible vectors of transmission to other vertebrates such as poultry, cattle, and humans (Cody et al., 2010; Waldenström et al., 2010). Migratory birds, due to their ability to cover long distances and fly freely are thought to be an important reservoir (Cerdà-Cuéllar et al., 2019). For example, the only foodborne illness outbreak traced back to a wild bird source occurred from migrating sandhill cranes stopping over at a pea farm in Alaska (REF). This ability to reach human-inhabited landscapes make it of special interest for public health related issued (Smith et al., 2020).

The role of wild birds in the spreading of pathogens in the Antarctica has received limited attention due to the region's isolation and relatively few exploitations by humans. However, some infectious agents may have invaded the region long before the arrival of humans due to migratory birds (Barbosa & Palacios, 2009; Cerdà-Cuéllar et al., 2019; Smith et al., 2020).

Skuas are seabirds from the genus *Stercorarius* that nest in the ground of both Arctic and Antarctic poles. Up to 95% of wintering skuas practice kleptoparasitism, a form of feeding in which skuas chase gulls, terns and other seabirds to steal their catches. Skuas are one of Antarctica's top avian predators, some even scavenge carcasses at breeding colonies of pinnipeds and penguins. Evidence of hybridization exists for all species; the largest overlap is found between the south polar skua (*Stercorarius maccormicki*) and brown skua (*Catharacta antarctica*) (González-Solís et al., 2017; Ritz et al., 2008).

1.3.1 South Polar Skua (*Stercorarius maccormicki*)

South polar skua, the smaller of the Antarctic skuas, have distinctive tan highlights in their feathers and tend to be more reserved (see Figure 2). First breeds start at age 5-6 years and usually the same birds share the same nest site every year after. One to two eggs are laid at a time, the eggs are olive to brown, blotched with darker brown. Even when both eggs hatch, only one young survive to fledging. Young are fed by both parents and leave nest soon after hatching (Hemmings, 2013a; Ritz et al., 2008).

Figure 2. South polar skua (*Stercorarius maccormicki*) (Audubon, n.d.)

South polar skua can be found far to the north in both Atlantic and Pacific oceans, most common in California during late spring and early fall where it remains far offshore. However, this predatory seabird only nests around the edges of the Antarctic continent both on islands and mainland (see Figure 3). The nests are usually close to penguin colonies, so the eggs and chicks can be fed. When the nests overlap with those of the larger brown skua, they take over control and the south polar skua must forage at sea. Nest site is on the ground scraped in soil or moss (Carneiro et al., 2014; Ritz et al., 2006).

Figure 3. Distribution map of south polar skua on the southern hemisphere (BirdLife International, 2020b)

1.3.2 Brown skua / subantarctic skua (*Catharacta Antarctica*)

The brown skua, also known as the subantarctic skua, is the biggest of the Antarctic skuas. It is dark brown with a very robust sharp falcon-like beak and tiny claws at the end of their webbed feet. Exhibits, during flight, a white patch on both upper and under wings (see Figure 4). The subspecies vary in plumage from largely brown to pale brown. Field separation from the south polar skua is difficult, even when the south polar has colder tones and smaller bill (Hemmings, 2013b; Ritz et al., 2008).

Figure 4. Brown / subantarctic skua (*Catharacta Antarctica*) (eBird, 2011)

Brown skuas hybridize with both south polar skuas and Chilean skuas, up to 12% of breeding pairs in populations within a wide hybrid zone consist of a female brown skua and a male south polar skua whose offspring can reproduce effectively (Ritz et al., 2008).

Brown skuas breed in the Antarctic and Sub Antarctic regions, but moves further north when not breeding (see Figure 5). As a breeding ritual, each skua pair has it breeding territory in the Antarctic that they occupy during the breeding season, where the pair scrapes a small depression in the ground to lay the eggs. During the incubation, one skua is sitting on the nest while the other looks for food, usually fish or carcasses from penguin colonies (González-Solís et al., 2017; Phillips et al., 2007).

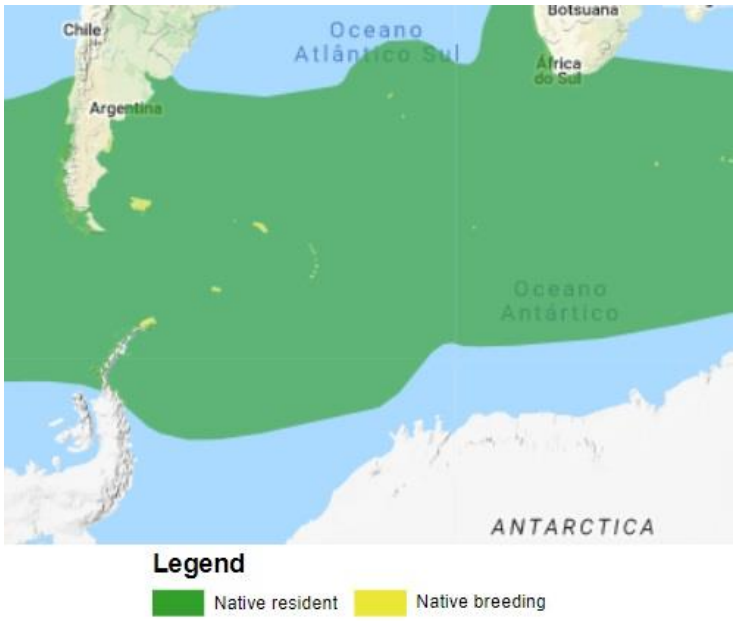


Figure 5. Distribution of brown skua on the Southern hemisphere (BirdLife International, 2020a)

1.4 Molecular epidemiology

Understanding the epidemiology of *Campylobacter spp* in wild birds is an essential part to study outbreaks. Campylobacteriosis are usually sporadic case and outbreaks are rarely notified and are difficult to investigate because it is possible that more than one strain coexists within the same individual. Another characteristic that poses a challenge when studying an outbreak is the genetic variability attributed to *Campylobacter,* that makes it possible for more than one

molecular profile to be present within the isolates of an outbreak (Cody et al., 2010; Mardones & López, 2017; WHO, 2012).

Even though the prevalence and genetic diversity of *Campylobacter spp* has been widely studied in humans and poultry, in wild birds is yet to be further studied. The characterization of *Campylobacter* is based in different phenotypic and genotypic markers. Conventional phenotypic methods such as morphology under the microscope, biochemical (catalase, oxidase, nitrate reductase) or serological tests are not so reliable and can be prone to false negatives. The use of molecular techniques is more reliable and specific. Polymerase chain reaction (PCR) allows the confirmation of *Campylobacter* species using different genes (Hubálek, 2004; Ranjbar et al., 2014).

For molecular epidemiology studies, restriction fragment length polymorphism (RFLP) has a high-resolution power. The molecular technique known as pulse field gel electrophoresis (PFGE) is also widely used and considered as the "gold standard" typing method (Ranjbar et al., 2014).

RFLP has been widely used to determine the genetic diversity of *Campylobacter*. In RFLP, a DNA sample is digested into fragments by restriction enzymes, resulting in restriction fragments that are then separated through electrophoresis according to their size, allowing to determine homologous DNA sequences, also known as polymorphisms (Bahmani et al., 2019; Steinhauserova et al., 2002). The *flaA*-RFLP technique consists in the amplification of the *flaA* flagellin gene through PCR and its digestion with Ddel enzyme to generate the profile. This technique is widely used in epidemiological studies for its simplicity and for its relative low cost. However, due to the possibility of a recombination between *flaA* and *flaB*, this technique is not suited for dynamic populations or long-term studies. Even though, *flaA*-RFLP can be a useful first step, especially when it is used along with other typing techniques such as PFGE (Taboada et al., 2013; Waldenström et al., 2010).

PFGE typing is a highly discriminative molecular technique used in epidemiological studies worldwide. It consists on the enzymatic digestion of the entire chromosomal DNA of the bacteria and the separation of the restriction fragments obtained using an electrical field of alternating polarity in different sizes (30-1100 bp). By comparing any two isolates, you can find out if they are clonal or not (Taboada et al., 2013). For *Campylobacter*, PFGE uses the enzymes *SmaI* and *KpnI* that have infrequent recognition sites and that is how it creates the profile. One

downside of this technique is that it is time consuming, require expensive equipment, and reproducibility can be difficult to achieve despite standardized protocols, especially in bacteria with genomic instability such as *C. jejuni* that can present different PFGE profiles after passing through the intestine of a host. Nevertheless, PFGE is still a reliable technique for short-term epidemiological analysis or for a high number of isolates (Bahmani et al., 2019; Shima et al., 2006).

Combining RFLP and PFGE techniques can provide a reliable source for characterization of *Campylobacter*. However, new technologies such as whole genome sequencing (WGS) by next generation sequencing (NGS) make possible to examine the entire genome of bacterial isolates and differentiate by a single nucleotide, providing the highest levels of accuracy for epidemiological studies. These technologies are becoming more accessible and applicable for a larger number of strains of interest (Forde & O'Toole, 2013).

2. Objectives

The aim of this study is to gain insight into the epidemiology of *C. lari* by determining its genetic diversity from isolates recovered from Brown skua (*Catharacta antarctica*) and South Polar skua (*Stercorarius maccormicki*) from different locations from the Southern Ocean.

3. Material and Methods

3.1 *C. lari* isolates

For the purpose of this study, *C. lari* isolation and identification had already been performed by the research team where I have developed the research project. A total of 231 *C. lari* isolates were recovered from cloacal swab samples of 55 skuas (brown skuas and south polar skuas) collected between September 2016 and February 2019. Of the total number of isolates, N=66 was of south polar skuas collected in Adelaide Is.; N=5 was of subantarctic skua collected in Gough Is.; N=32 was of brown skuas collected at Gough Base in Gough Is, N=58 was of brown skuas collected in Svarthamaren; and N=70 was of brown skuas collected in Falkland Is. (see Figure 6).

Figure 6. Map of the localization of Adelaide, Gough, Svarthamaren and Falkland Islands (Google maps)

3.2 DNA extraction

DNA extraction from the *C. lari* isolates was performed using 100µl of InstaGene Matrix (BioRad) and adding 250 µl of the bacterial suspension in PBS and mixed using vortex. The suspension was then incubated in the bath at 56ºC for 30 min and vortexed at high speed for 10 seconds, the suspension was then placed at 99ºC in the thermoblock for 8 minutes. After the

time has passed, the Eppendorf tubes with the mixture were centrifuged at 13000 rpm for 3 min. Finally, 200 µl of the supernatant was collected into new tubes, labelled correctly and stored at -20ºC until use.

3.3 PCR-*FlaA*

FlaA gene amplicon was obtained using PCR. DNA was first quantified using BioDrop and appropriate dilutions were prepared using MiliQ water, so each sample had the same DNA concentration (20ng/µl). The primers used for the amplification of flaA gene of *C. lari* are shown in Table 1 and PCR conditions are specified in Table 2.

Table 1. Primers used for the amplification of *flaA* in *C. lari*

Primers	*Sequence (5'3')*
Lari A-f (forward)	GGA TTT CGT ATA AAT ACT AAT GTG GC
Lari A-r (reverse)	TTG TAA TAA TCT CAT TAC ATT TTG

Table 2. Reaction mix and conditions for the *flaA* amplification in *C. lari*

PCR flaA	**Reaction MIX**
	20µl H2Od
	25µl Master Mix Promega 2X
	2µl primer Lari A-f 10µM (1/10)
	2µl primer Lari A-r 10µM (1/10)
	Total MIX (Reaction volume 50µl)
	MIX 47,5µl
	DNA 2,5µl
	PCR program:
	94ºC, 5' // 94ºC, 1' / 45ºC, 1' / 72ºC, 1'30'' // 72ºC, 7' // 4ºC, ∞ (30 cycles)

Electrophoresis was used to confirm if the flaA amplicon was obtained from the PCR (Figure 7). An agarose gel (100ml agarose 1.8% TAE + 180µl Etidium Bromide diluted 1/100) was run at 120V for 30 minutes; 8µl of the sample (flaA amplicon mixed with blue dye) and 3µl of the molecular weight marker 2-log DNA ladder were loaded into the gel and visualized using UV light. The flaA amplicon of *C. lari* has a molecular weight of 1700bp.

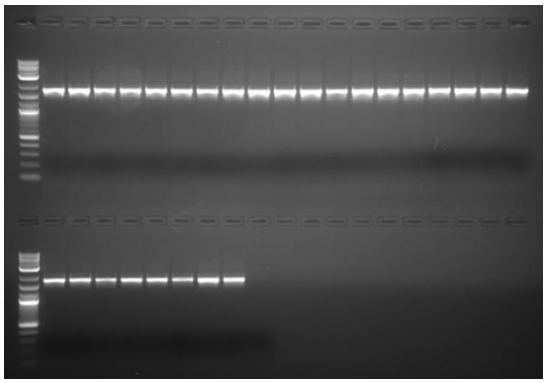


Figure 7. Agarose gel electrophoresis of flaA amplicons of *C. lari*

All isolates that showed flaA amplicon were analysed by RFLP and a selection of those were further analysed by PFGE. Those samples that did not show flaA PCR amplicon were listed directly for PFGE.

3.4 *flaA*-RFLP (Restriction Fragment Length Polymorphism)

The amplified product of flaA PCR fragment (1700bp) was digested using the restriction enzyme DdeI (HypF3I; FastDigest®, Thermo Fisher Scientific, Waltham, MA, USA), conditions used are shown in Table 3 below.

Table 3. Reaction mix for the *flaA*-RFLP enzymatic digestion

Reaction MIX:
12µl sterile H_2O
2µl Buffer 2X (Green Dye)
1µl Ddel enzyme (HypF3I)
Total MIX **(20µl)**:
MIX 15µl
DNA amplicon *flaA* 5µl
Digestion:
10 minutes at 37ºC
Inactivation:
5 minutes at 65ºC

Once the enzymatic digestion was completed, the products were separated by electrophoresis on a 2.5% agarose gel in 1x TAE buffer at 90V for 3h, 20µl of the sample and 3µl of the molecular weight marker 2-log DNA ladder were loaded into the gel, UV light was used to visualize the bands (Figure 8).

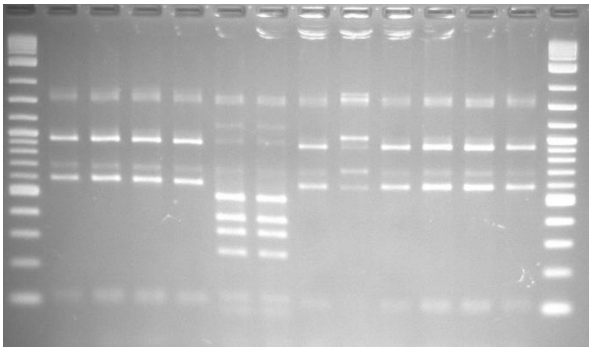


Figure 8. Agarose gel of *flaA*-RFLP enzyme digestion

Band patterns resulting from *flaA*-RFLP were used to determine the PFGE selection. Isolates from the same individual with the same *flaA*-RFLP profile were considered as the same strain and only one of them was selected for PFGE.

3.5 Pulsed Field Gel Electrophoresis (PFGE)

PFGE was performed according to the standard operating procedure described in PulseNet (https://pulsenetinternational.org/protocols/pfge/). Isolates were digested using the SmaI restriction enzyme. Molecular weight marker used was *Salmonella braenderup* digested with XbaI enzyme. Electrophoresis was performed with a Chef-DR III system (BioRad, Hercules, CA, USA). The PFGE gel was dyed using EtBr and UV light was used to visualize the bands (Figure 9).

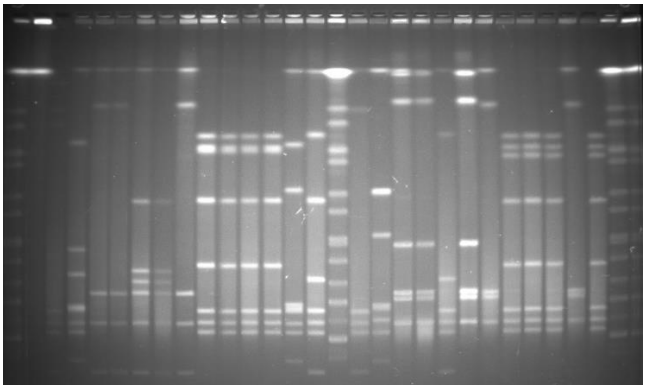


Figure 9. Pulse Field Gel Electrophoresis band pattern

3.6 Band patterns comparison and analysis

RFLP and PFGE band patterns were analyzed using Fingerprinting II v3.0 software (Bio-Rad, Hercules, CA, USA). Similarity matrices were calculated using the Dice coefficient with tolerance and optimization values of 1.0%. Afterwards, dendrograms were constructed for both RFLP and PFGE based on an unweighted-pair group method with arithmetic mean (UPGMA) cluster analysis. A cut-off of 90% was used for the determination of the different profiles.

4. Results

We used flaA-RFLP in the first instance to determine the genetic diversity among *Campylobacter* isolates within an individual host and within a colony, and lastly to determine similarities among localities. Overall, 231 isolates of *C. lari* from 14 south polar skuas, 2 subantarctic skuas and 39 brown skuas were genotyped using *flaA*-RFLP, and 19 different profiles (cut off, 90% similarity) were identified (Figure 10). In most cases, each bird carried a single profile, but in some instances also the same profile was found among different individuals from the same colony. More interestingly, same profiles were also found among individuals from different localities (Adelaide Is and Gough Is) and bird species (brown skuas, south polar skuas). When *C. lari* isolates from marine birds (mainly skuas) from other localities from the Southern Ocean, previously characterized by RFLP and available in the database of the research group were added to the analyses, the number of localities were the same strain (=same profile) was found increased (Figure 11). Thus, same strain could be found in 2 (e.g. Svarthamaren, Kerguelen; Adelaide, Kerguelen; Adelaide, Gough), 3 (e.g. King George Is, Svarthamaren Falkland; Gough, Svarthamaren, Falkland), 4 (e.g. Adelaide, Falkland, Crozet Kerguelen Is), 5 (e.g. Adelaide, King George Is, Falkland, Gough Is, Crozet) or up to 6 (e.g. Adelaide, King George Is, Gough Is, Crozet, Kerguelen Is., Amsterdam Is) different localities.

The results from the *flaA*-RFLP were used to select the isolates that were suited for PFGE. In the cases where isolates from the same individual showed the same RFLP profile only one of the isolates were selected for PFGE. Four isolates from the same individual that did not show flaA amplification (PCR) nor RFLP profile were also included in the PFGE. Overall, 37 isolates

of *C. lari* from 15 south polar skuas, 2 subantarctic skuas and 9 brown skuas were genotyped by PFGE, 27 different profiles were identified (Figure 12). From the comparison between flaA-RFLP to PFGE two pulsotypes of isolates with the same RFLP (i.e. RFLP profile Nº 4 and 14) have also the same PFGE profile, and only one of them have isolates from different localities. It is interesting to mention that these profiles that are consistent in RFLP and PFGE showed a higher number of bands in RFLP which might contribute to a more accurate result. These differences might be explained by the higher discriminatory power that is attributed to PFGE.

In order to increase the scope of this study, further analysis of PFGE of *C. lari* from a diverse variety of localities that was available in the database of the research group was added to the study (Figure 13). The same strains could be found in 2 (Marion, Kerguelen Is.), 3 (Livingston, Gough, Marion), 4 (Adelaide, Gough Is, Cochons, Amsterdam), and up to 6 (Livingston, Falkland Is, Gough Is, Strandfontain, Marion, Amsterdam Is.) different localities.

Figure 10. flaA-RFLP dendrogram of *C. lari* isolates from brown skua, subantarctic skua, and south polar skua from Gough and Adelaide Islands.

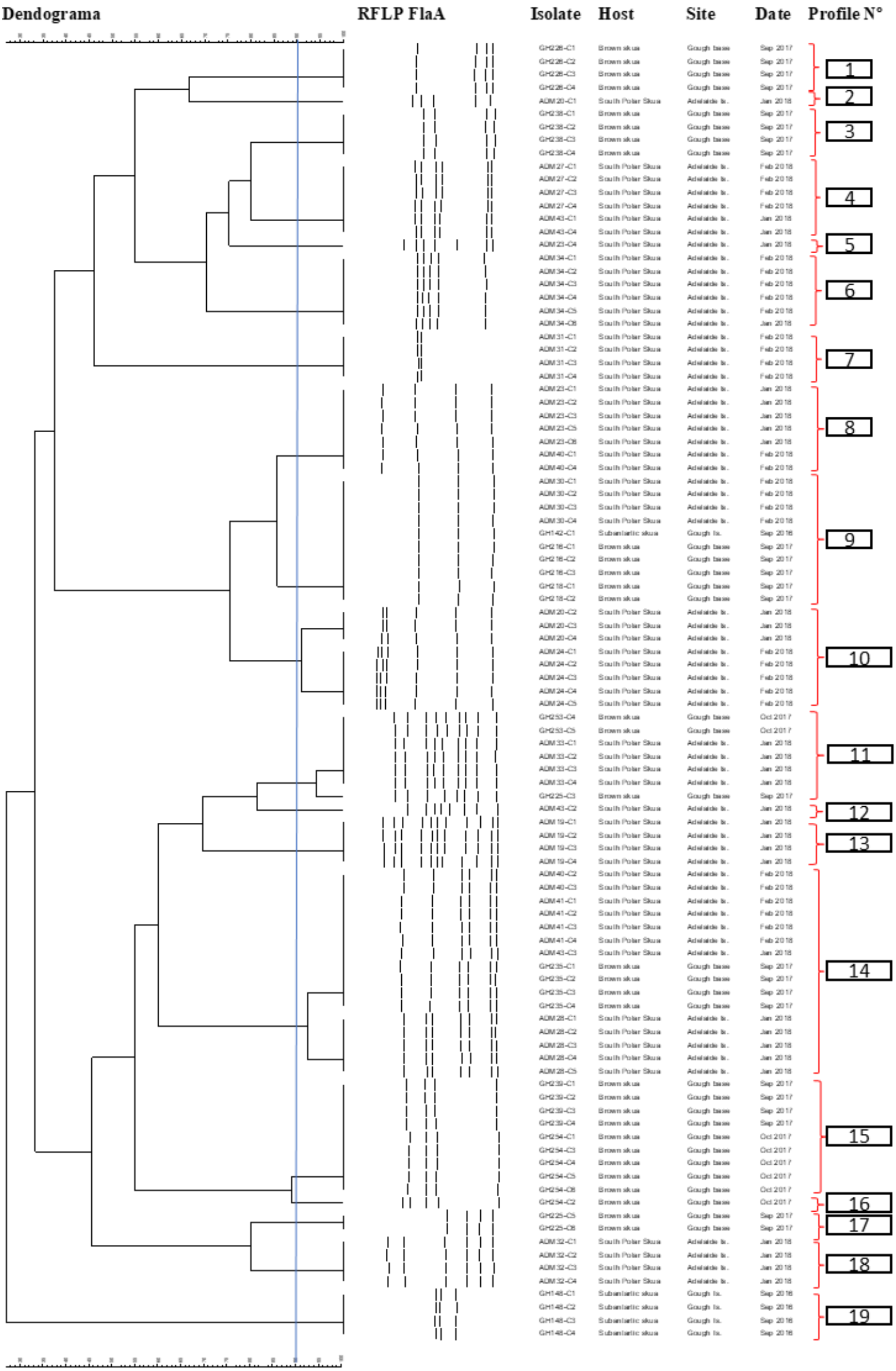
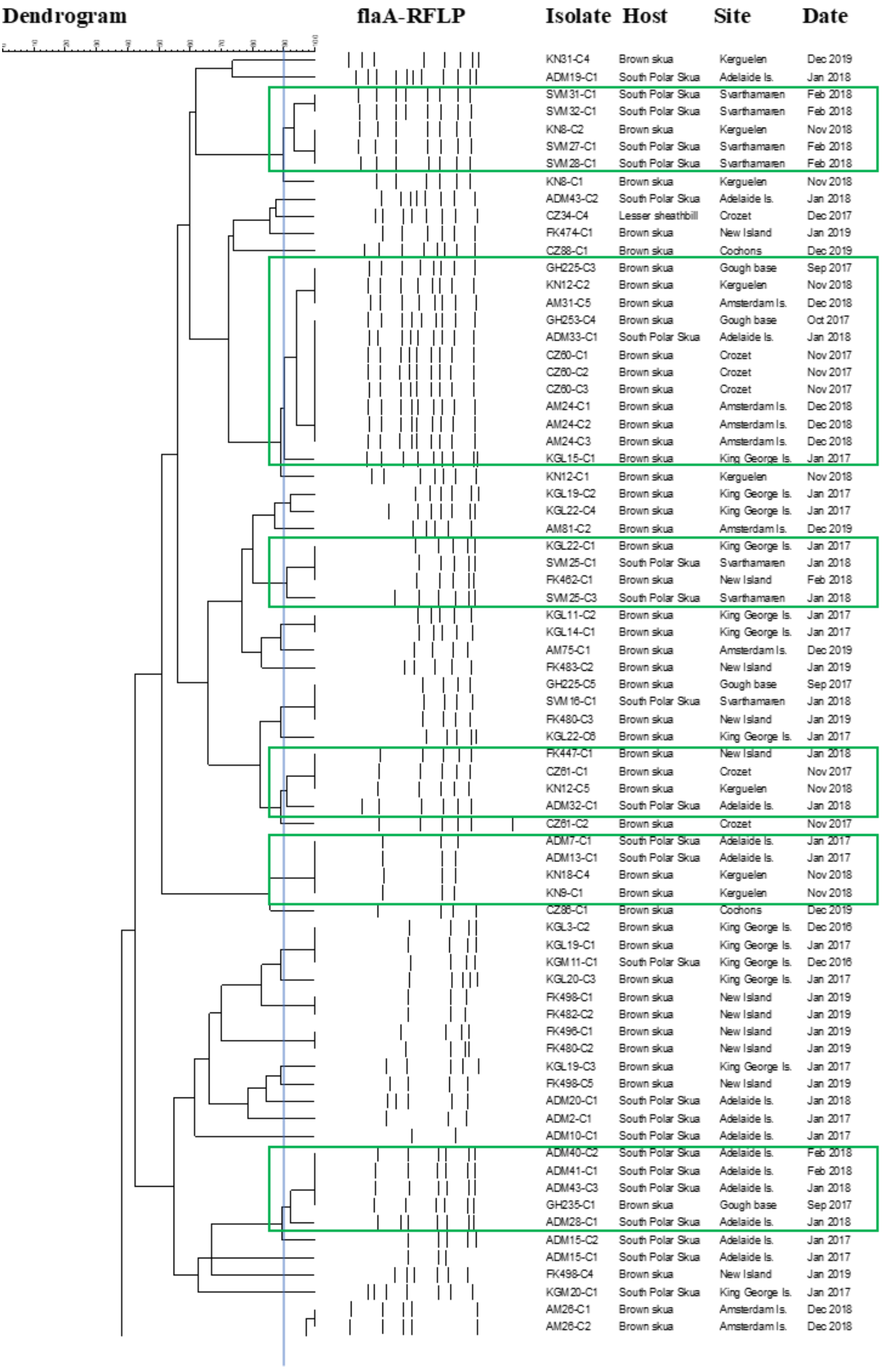
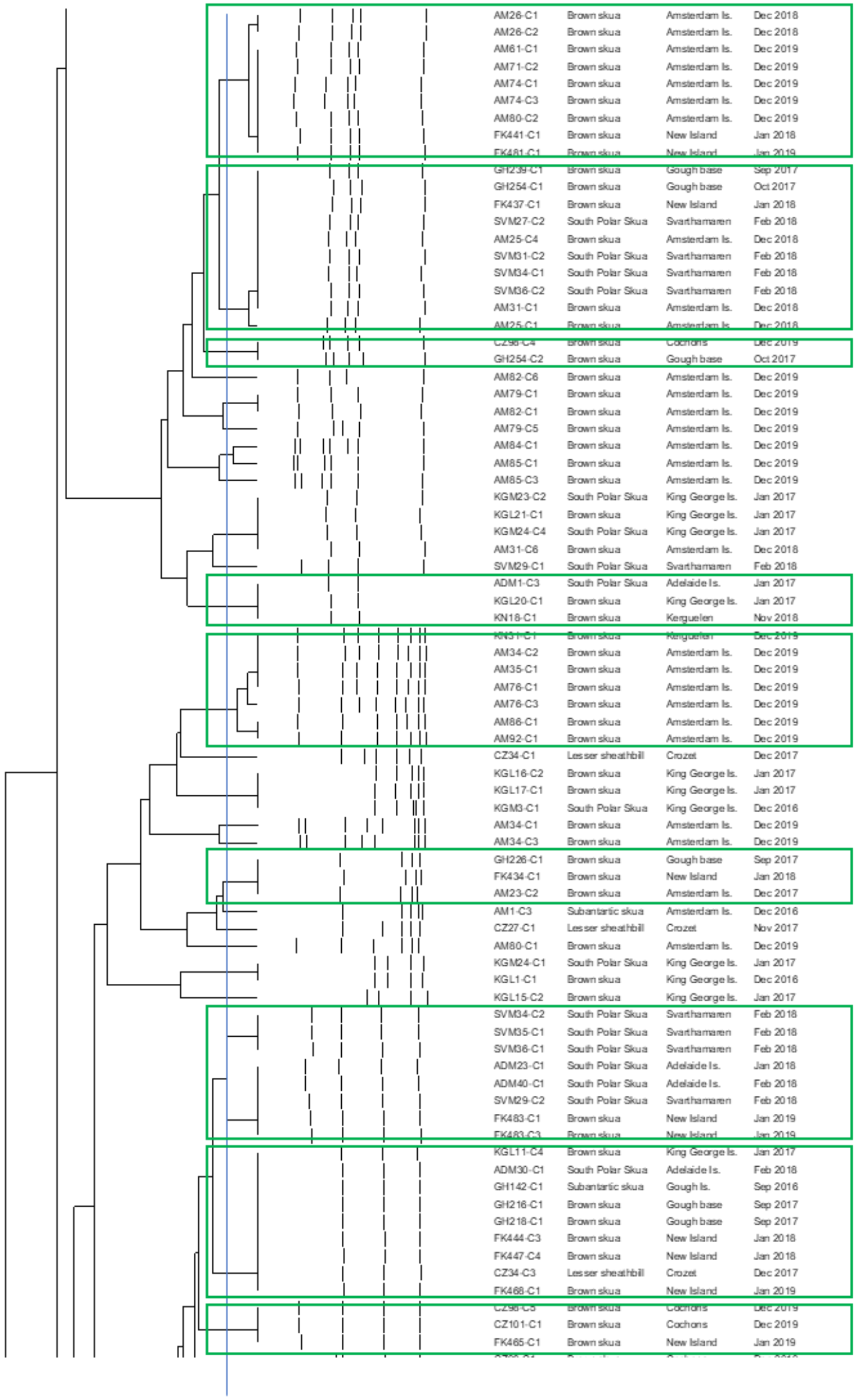


Figure 11. f*laA*-RFLP dendrogram of *C. lari* isolates including data retrieved from own database. Green squares highlight isolates from different localities showing >90% similarity.




AM26-C1 Brown skua Amsterdam Is. Dec 2018
AM26-C2 Brown skua Amsterdam Is. Dec 2018
AM61-C1 Brown skua Amsterdam Is. Dec 2019
AM71-C2 Brown skua Amsterdam Is. Dec 2019
AM74-C1 Brown skua Amsterdam Is. Dec 2019
AM74-C3 Brown skua Amsterdam Is. Dec 2019
AM80-C2 Brown skua Amsterdam Is. Dec 2019
FK441-C1 Brown skua New Island Jan 2018
FK481-C1 Brown skua New Island Jan 2019
GH239-C1 Brown skua Gough base Sep 2017
GH254-C1 Brown skua Gough base Oct 2017
FK437-C1 Brown skua New Island Jan 2018
SVM27-C2 South Polar Skua Svarthamaren Feb 2018
AM25-C4 Brown skua Amsterdam Is. Dec 2018
SVM31-C2 South Polar Skua Svarthamaren Feb 2018
SVM34-C1 South Polar Skua Svarthamaren Feb 2018
SVM36-C2 South Polar Skua Svarthamaren Feb 2018
AM31-C1 Brown skua Amsterdam Is. Dec 2018
AM25-C1 Brown skua Amsterdam Is. Dec 2018
CZ96-C4 Brown skua Cochons Dec 2019
GH254-C2 Brown skua Gough base Oct 2017
AM82-C6 Brown skua Amsterdam Is. Dec 2019
AM79-C1 Brown skua Amsterdam Is. Dec 2019
AM82-C1 Brown skua Amsterdam Is. Dec 2019
AM79-C5 Brown skua Amsterdam Is. Dec 2019
AM84-C1 Brown skua Amsterdam Is. Dec 2019
AM85-C1 Brown skua Amsterdam Is. Dec 2019
AM85-C3 Brown skua Amsterdam Is. Dec 2019
KGM23-C2 South Polar Skua King George Is. Jan 2017
KGL21-C1 Brown skua King George Is. Jan 2017
KGM24-C4 South Polar Skua King George Is. Jan 2017
AM31-C6 Brown skua Amsterdam Is. Dec 2018
SVM29-C1 South Polar Skua Svarthamaren Feb 2018
ADM1-C3 South Polar Skua Adelaide Is. Jan 2017
KGL20-C1 Brown skua King George Is. Jan 2017
KN18-C1 Brown skua Kerguelen Nov 2018
KN31-C1 Brown skua Kerguelen Dec 2019
AM34-C2 Brown skua Amsterdam Is. Dec 2019
AM35-C1 Brown skua Amsterdam Is. Dec 2019
AM76-C1 Brown skua Amsterdam Is. Dec 2019
AM76-C3 Brown skua Amsterdam Is. Dec 2019
AM86-C1 Brown skua Amsterdam Is. Dec 2019
AM92-C1 Brown skua Amsterdam Is. Dec 2019
CZ34-C1 Lesser sheathbill Crozet Dec 2017
KGL16-C2 Brown skua King George Is. Jan 2017
KGL17-C1 Brown skua King George Is. Jan 2017
KGM3-C1 South Polar Skua King George Is. Dec 2016
AM34-C1 Brown skua Amsterdam Is. Dec 2019
AM34-C3 Brown skua Amsterdam Is. Dec 2019
GH226-C1 Brown skua Gough base Sep 2017
FK434-C1 Brown skua New Island Jan 2018
AM23-C2 Brown skua Amsterdam Is. Dec 2017
AM1-C3 Subantartic skua Amsterdam Is. Dec 2016
CZ27-C1 Lesser sheathbill Crozet Nov 2017
AM80-C1 Brown skua Amsterdam Is. Dec 2019
KGM24-C1 South Polar Skua King George Is. Jan 2017
KGL1-C1 Brown skua King George Is. Dec 2016
KGL15-C2 Brown skua King George Is. Jan 2017
SVM34-C2 South Polar Skua Svarthamaren Feb 2018
SVM35-C1 South Polar Skua Svarthamaren Feb 2018
SVM36-C1 South Polar Skua Svarthamaren Feb 2018
ADM23-C1 South Polar Skua Adelaide Is. Jan 2018
ADM40-C1 South Polar Skua Adelaide Is. Feb 2018
SVM29-C2 South Polar Skua Svarthamaren Feb 2018
FK483-C1 Brown skua New Island Jan 2019
FK483-C3 Brown skua New Island Jan 2019
KGL11-C4 Brown skua King George Is. Jan 2017
ADM30-C1 South Polar Skua Adelaide Is. Feb 2018
GH142-C1 Subantartic skua Gough Is. Sep 2016
GH216-C1 Brown skua Gough base Sep 2017
GH218-C1 Brown skua Gough base Sep 2017
FK444-C3 Brown skua New Island Jan 2018
FK447-C4 Brown skua New Island Jan 2018
CZ34-C3 Lesser sheathbill Crozet Dec 2017
FK468-C1 Brown skua New Island Jan 2019
CZ96-C5 Brown skua Cochons Dec 2019
CZ101-C1 Brown skua Cochons Dec 2019
FK465-C1 Brown skua New Island Jan 2019

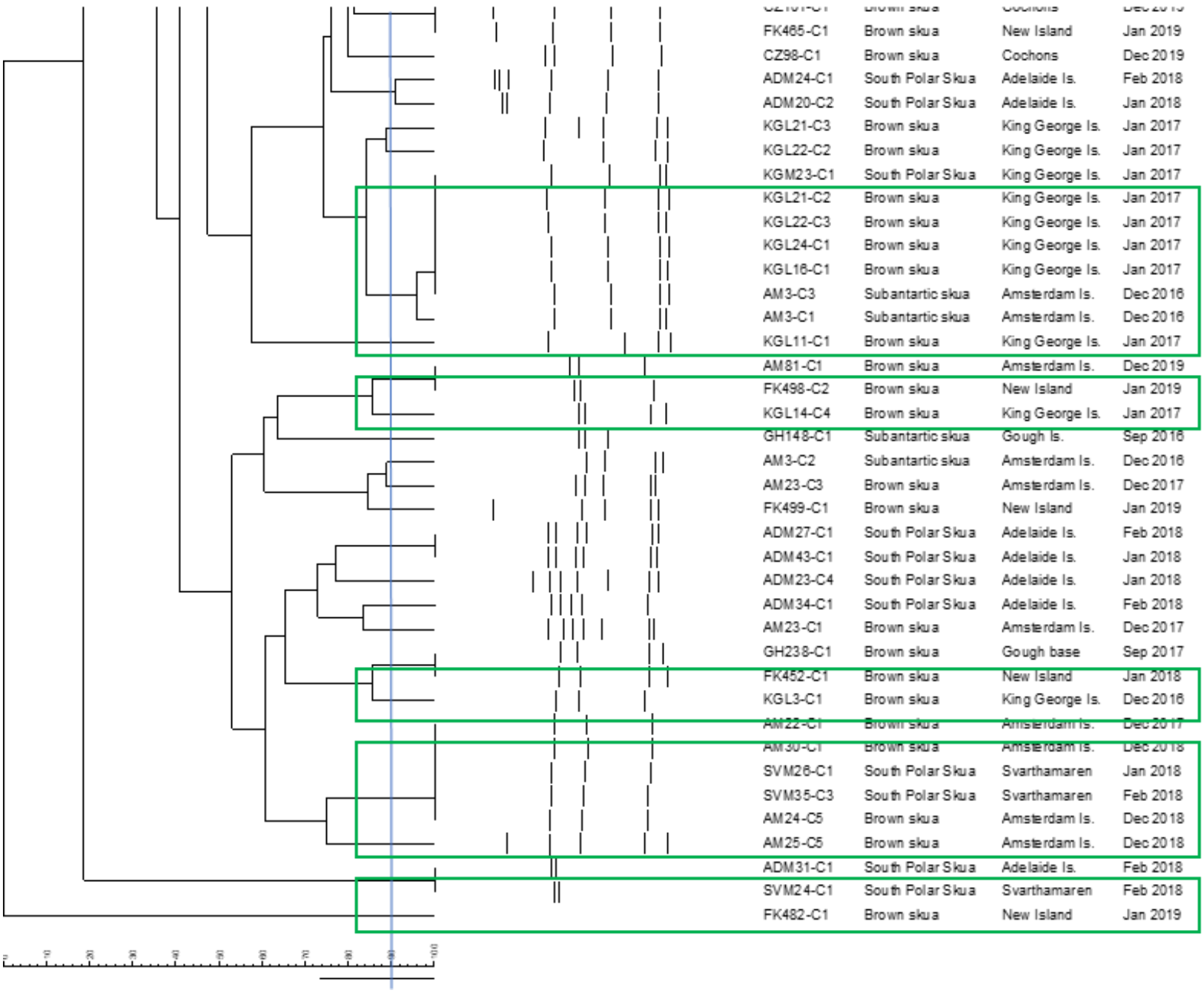
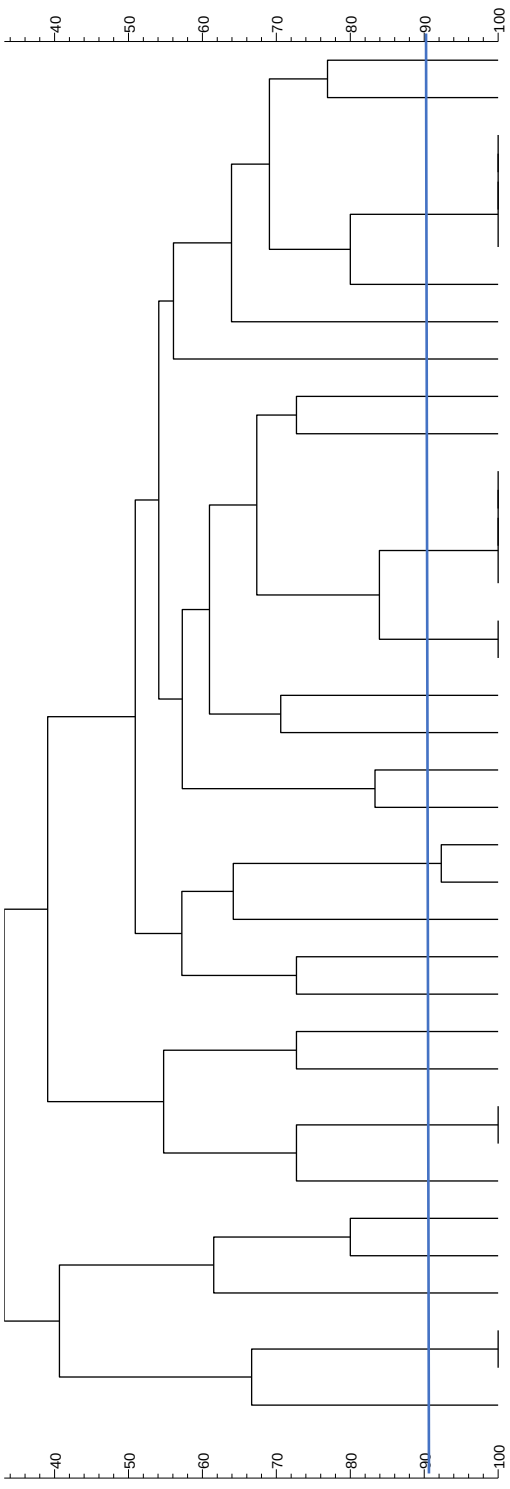
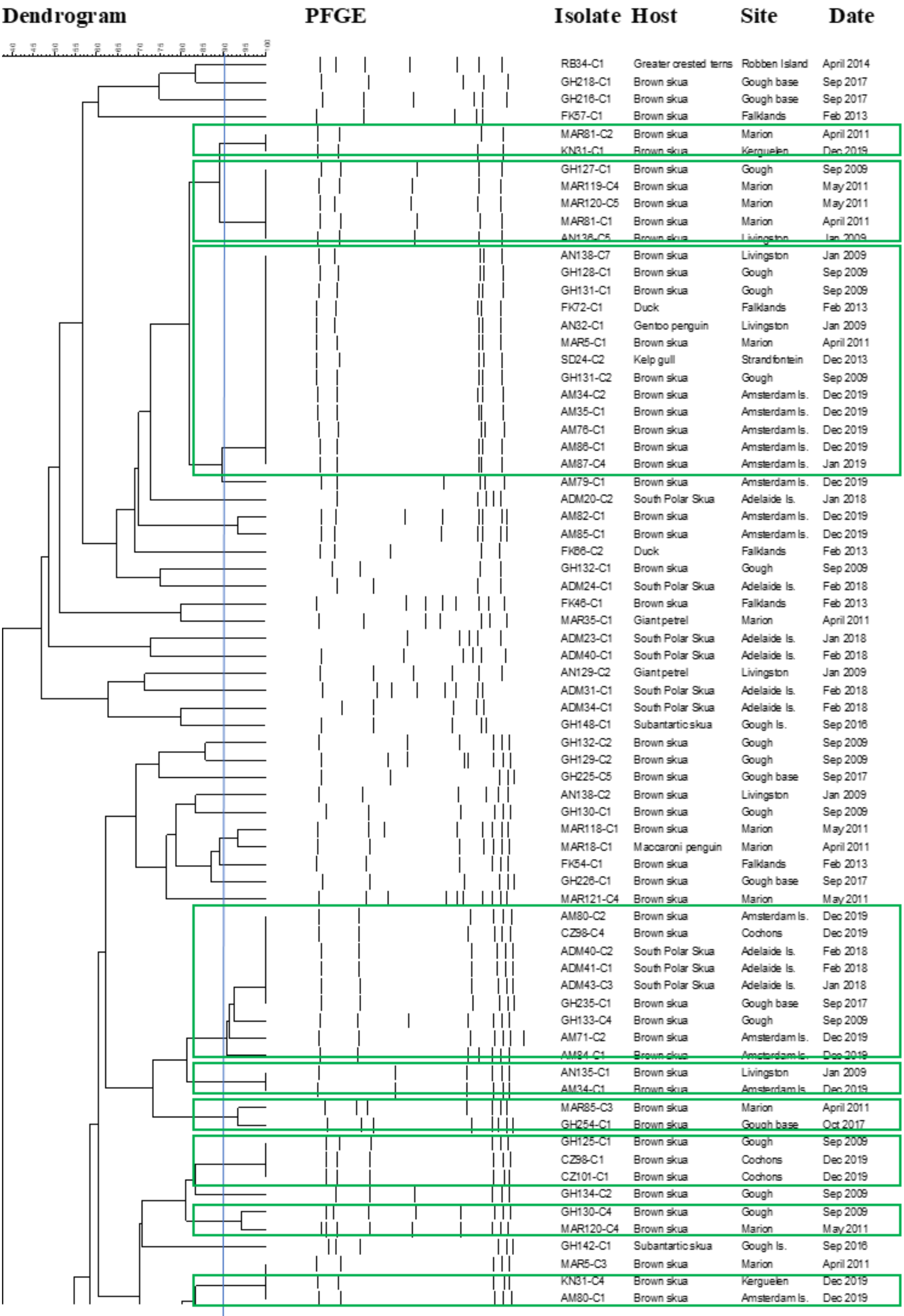

FK465-C1 Brown skua New Island Jan 2019
CZ98-C1 Brown skua Cochons Dec 2019
ADM24-C1 South Polar Skua Adelaide Is. Feb 2018
ADM20-C2 South Polar Skua Adelaide Is. Jan 2018
KGL21-C3 Brown skua King George Is. Jan 2017
KGL22-C2 Brown skua King George Is. Jan 2017
KGM23-C1 South Polar Skua King George Is. Jan 2017
KGL21-C2 Brown skua King George Is. Jan 2017
KGL22-C3 Brown skua King George Is. Jan 2017
KGL24-C1 Brown skua King George Is. Jan 2017
KGL16-C1 Brown skua King George Is. Jan 2017
AM3-C3 Subantartic skua Amsterdam Is. Dec 2016
AM3-C1 Subantartic skua Amsterdam Is. Dec 2016
KGL11-C1 Brown skua King George Is. Jan 2017
AM81-C1 Brown skua Amsterdam Is. Dec 2019
FK498-C2 Brown skua New Island Jan 2019
KGL14-C4 Brown skua King George Is. Jan 2017
GH148-C1 Subantartic skua Gough Is. Sep 2016
AM3-C2 Subantartic skua Amsterdam Is. Dec 2016
AM23-C3 Brown skua Amsterdam Is. Dec 2017
FK499-C1 Brown skua New Island Jan 2019
ADM27-C1 South Polar Skua Adelaide Is. Feb 2018
ADM43-C1 South Polar Skua Adelaide Is. Jan 2018
ADM23-C4 South Polar Skua Adelaide Is. Jan 2018
ADM34-C1 South Polar Skua Adelaide Is. Feb 2018
AM23-C1 Brown skua Amsterdam Is. Dec 2017
GH238-C1 Brown skua Gough base Sep 2017
FK452-C1 Brown skua New Island Jan 2018
KGL3-C1 Brown skua King George Is. Dec 2016
AM22-C1 Brown skua Amsterdam Is. Dec 2017
AM30-C1 Brown skua Amsterdam Is. Dec 2018
SVM26-C1 South Polar Skua Svarthamaren Jan 2018
SVM35-C3 South Polar Skua Svarthamaren Feb 2018
AM24-C5 Brown skua Amsterdam Is. Dec 2018
AM25-C5 Brown skua Amsterdam Is. Dec 2018
ADM31-C1 South Polar Skua Adelaide Is. Feb 2018
SVM24-C1 South Polar Skua Svarthamaren Feb 2018
FK482-C1 Brown skua New Island Jan 2019

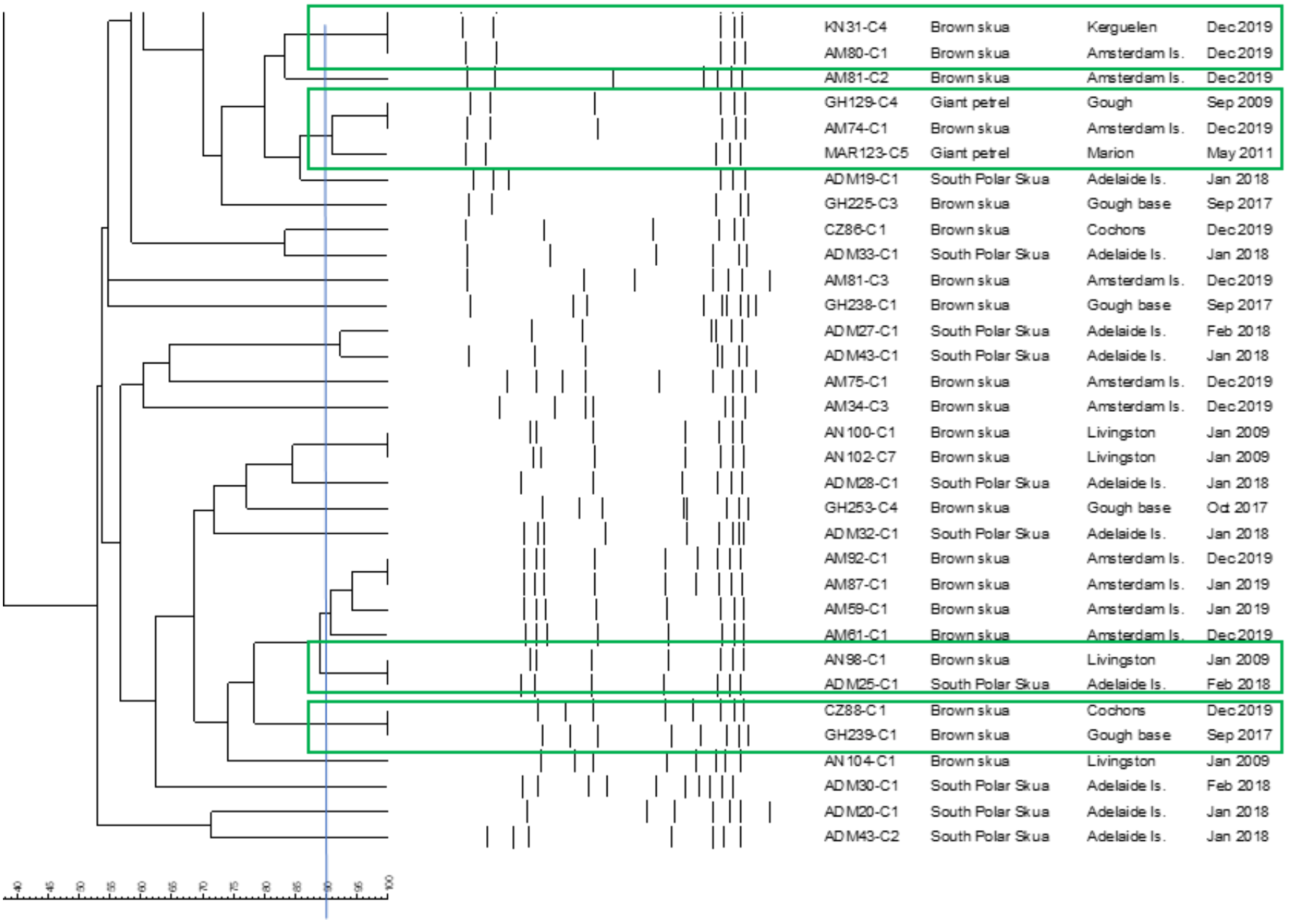
Figure 12. PFGE dendrogram of *C. lari* isolates from brown skua, subantarctic skua, and south polar skua from Gough and Adelaide Islands



Isolate	Host	Site	Date	Profile Nº
ADM28-C1	South Polar Skua	Adelaide Is.	Jan 2018	1
ADM43-C2	South Polar Skua	Adelaide Is.	Jan 2018	2
ADM25-C1	South Polar Skua	Adelaide Is.	Feb 2018	3
ADM25-C2	South Polar Skua	Adelaide Is.	Feb 2018	
ADM25-C3	South Polar Skua	Adelaide Is.	Feb 2018	
ADM25-C4	South Polar Skua	Adelaide Is.	Feb 2018	
GH239-C1	Brown skua	Gough base	Sep 2017	4
ADM30-C1	South Polar Skua	Adelaide Is.	Feb 2018	5
ADM20-C1	South Polar Skua	Adelaide Is.	Jan 2018	6
GH225-C5	Brown skua	Gough base	Sep 2017	7
GH226-C1	Brown skua	Gough base	Sep 2017	8
ADM40-C2	South Polar Skua	Adelaide Is.	Feb 2018	9
ADM41-C1	South Polar Skua	Adelaide Is.	Feb 2018	
ADM43-C3	South Polar Skua	Adelaide Is.	Jan 2018	
GH235-C1	Brown skua	Gough base	Sep 2017	
GH254-C1	Brown skua	Gough base	Oct 2017	10
GH254-C2	Brown skua	Gough base	Oct 2017	
GH253-C4	Brown skua	Gough base	Oct 2017	11
ADM32-C1	South Polar Skua	Adelaide Is.	Jan 2018	12
ADM19-C1	South Polar Skua	Adelaide Is.	Jan 2018	13
GH142-C1	Subantartic skua	Gough Is.	Sep 2016	14
ADM27-C1	South Polar Skua	Adelaide Is.	Feb 2018	15
ADM43-C1	South Polar Skua	Adelaide Is.	Jan 2018	
GH238-C1	Brown skua	Gough base	Sep 2017	16
ADM33-C1	South Polar Skua	Adelaide Is.	Jan 2018	17
GH225-C3	Brown skua	Gough base	Sep 2017	18
GH216-C1	Brown skua	Gough base	Sep 2017	19
GH218-C1	Brown skua	Gough base	Sep 2017	20
ADM23-C1	South Polar Skua	Adelaide Is.	Jan 2018	21
ADM23-C4	South Polar Skua	Adelaide Is.	Jan 2018	
ADM40-C1	South Polar Skua	Adelaide Is.	Feb 2018	22
ADM34-C1	South Polar Skua	Adelaide Is.	Feb 2018	23
GH148-C1	Subantartic skua	Gough Is.	Sep 2016	24
ADM31-C1	South Polar Skua	Adelaide Is.	Feb 2018	25
ADM20-C2	South Polar Skua	Adelaide Is.	Jan 2018	26
ADM20-C3	South Polar Skua	Adelaide Is.	Jan 2018	
ADM24-C1	South Polar Skua	Adelaide Is.	Feb 2018	27

Figure 13. PFGE dendrogram of *C. lari* isolates including data retrieved from own database. Green squares highlight isolates from different localities showing >90% similarity.




KN31-C4 Brown skua Kerguelen Dec 2019
AM80-C1 Brown skua Amsterdam Is. Dec 2019
AM81-C2 Brown skua Amsterdam Is. Dec 2019
GH129-C4 Giant petrel Gough Sep 2009
AM74-C1 Brown skua Amsterdam Is. Dec 2019
MAR123-C5 Giant petrel Marion May 2011
ADM19-C1 South Polar Skua Adelaide Is. Jan 2018
GH225-C3 Brown skua Gough base Sep 2017
CZ86-C1 Brown skua Cochons Dec 2019
ADM33-C1 South Polar Skua Adelaide Is. Jan 2018
AM81-C3 Brown skua Amsterdam Is. Dec 2019
GH238-C1 Brown skua Gough base Sep 2017
ADM27-C1 South Polar Skua Adelaide Is. Feb 2018
ADM43-C1 South Polar Skua Adelaide Is. Jan 2018
AM75-C1 Brown skua Amsterdam Is. Dec 2019
AM34-C3 Brown skua Amsterdam Is. Dec 2019
AN100-C1 Brown skua Livingston Jan 2009
AN102-C7 Brown skua Livingston Jan 2009
ADM28-C1 South Polar Skua Adelaide Is. Jan 2018
GH253-C4 Brown skua Gough base Oct 2017
ADM32-C1 South Polar Skua Adelaide Is. Jan 2018
AM92-C1 Brown skua Amsterdam Is. Dec 2019
AM87-C1 Brown skua Amsterdam Is. Jan 2019
AM59-C1 Brown skua Amsterdam Is. Jan 2019
AM61-C1 Brown skua Amsterdam Is. Dec 2019
AN98-C1 Brown skua Livingston Jan 2009
ADM25-C1 South Polar Skua Adelaide Is. Feb 2018
CZ88-C1 Brown skua Cochons Dec 2019
GH239-C1 Brown skua Gough base Sep 2017
AN104-C1 Brown skua Livingston Jan 2009
ADM30-C1 South Polar Skua Adelaide Is. Feb 2018
ADM20-C1 South Polar Skua Adelaide Is. Jan 2018
ADM43-C2 South Polar Skua Adelaide Is. Jan 2018

5. Discussion

Campylobacteriosis is the most reported gastrointestinal disease in humans. This specie can invade the organisms of warm blood animals, including humans and specially birds due to their blood temperature (Chlebicz & Śliżewska, 2018). Migratory birds might be key for a better understanding and management of emerging infectious diseases (Barbosa & Palacios, 2009; Cerdà-Cuéllar et al., 2019). In this study, we provided an insight of the epidemiology of *Campylobacter lari* in brown and south polar skuas from the Southern Ocean and Antarctica.

The genetic diversity of *C. lari* was assessed using *flaA*-RFLP and PFGE. Both typing techniques are widely used in epidemiological studies. PFGE showed a higher genetic diversity against flaA-RFLP, this might be explained by the highly discriminative level of PFGE and also to the high genetic instability of the *Campylobacter* genome (Waldenström et al., 2010). It is of especial interest to mention that those profiles that were the same in RFLP but different in PFGE coincide to profiles with fewer bands in RFLP, while the profiles that were the same both in RFLP and PFGE had more complete band patterns, this can be explained because fewer bands relate to a lower discriminative capacity. These variations in the typing techniques reinforce the importance to use at least two techniques, like RFLP and PFGE, to confirm the results. Furthermore, to prove the results of the *C. lari* profiles obtained in this study, the use of a second enzyme (i.e. KpnI) might be useful. The use of other techniques such as MLST have also proven to provide more accurate results when used along with PFGE. (Bahmani et al., 2019; Forde & O'Toole, 2013; Taboada et al., 2013)

The incorporation of previously characterized isolates to the RFLP and PFGE analyses proved that many localities shared the same profiles. Wild birds are very mobile, and thus can carry pathogens through large distances. Both brown skuas and south polar skuas can travel long distances and from one island from the Southern Ocean to the other (Smith et al., 2020). Brown skuas breed across a long latitudinal range from the Antarctic and South ocean to temperate regions. Individuals often use a migration strategy which they follow year after year. However, the variability of migration strategies among individuals and populations is remarkable, which makes it difficult to establish a single pattern. Also, post-breeding movements might also vary from true long distances like the Benguela current in the Atlantic Ocean to staying in the vicinity of breeding grounds. Males usually have shorter migration than the females. As for the south polar skua, it is also known that travel long distances during the wintering that can reach up to

California. This skua can be both in the islands and in mainland. This intense migrations and ability to communicate from island to island explain why there are *C. lari* isolates that share the same profile even through the distance (Delord et al., 2018; Krietsch et al., 2017; Ritz et al., 2008).

Migration is considered a response to seasonal variation, in specific to prey availability. During breeding season both south polar and brown skuas tend to build nest near colonies of penguins, and outside of the breeding period can range through much larger areas. This might provide an insight of the broad molecular variability of *C. lari* in these birds as well as why some share the same pulsotype (Cody et al., 2010; Delord et al., 2018; Schultz et al., 2018).

No special host specificity was observed. Even though there are certain profiles that are shared by only one individual, there was also cases in which the same profile was shared by different individuals and both by brown and south polar skuas. The overlap of species (South Polar skua and Brown skua) that was found can be explained by the distribution of this taxa, in Figure 14 it can be observed an extensive area of hybridization between South Polar and Brown skuas. These birds have shown an extensive variation during the post breeding movement. Up to 12% of breeding pairs in populations within a wide hybrid zone consist of a female brown skua and a male south polar skua whose offspring can reproduce effectively. This hybridization has also made more challenging the distinction of one skua from the other (Ritz et al., 2006; Smith et al., 2020).

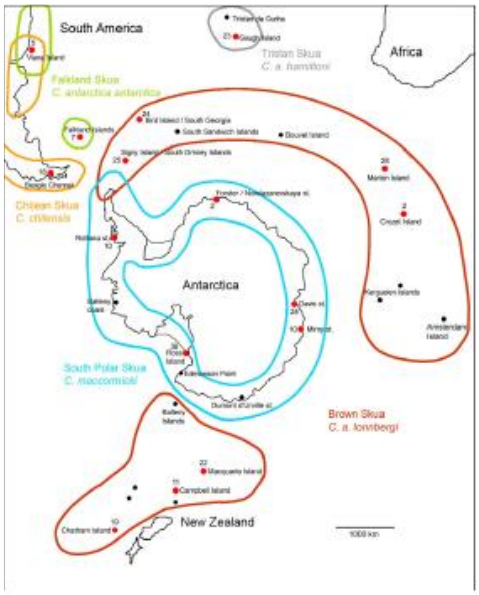


Figure 14. Distribution of the five southern taxa skua (Ritz et al., 2008)

This connectivity between the islands and the skuas pose a risk for the potential spread of pathogens. Wild birds can exchange diseases with domestic birds, other animals such as pigs or cattle, and humans. To establish a better understanding between wild birds and human diseases is important to keep working towards understanding transmission (Barbosa & Palacios, 2009; Jones et al., 2008; Karesh et al., 2012). It has been established that pathogens can be dispersed by migratory birds. Nonetheless, another reason of why studying this transmission is important is that it has recently been stablished that humans have also contributed to their dispersal. In the antarctica, reports of wildlife suggested the spread of enteric bacteria from people to seabirds. Recurring outbreaks of emerging and re-emerging zoonoses are a reminder that health of humans, animals and the environment are interconnected, an interdisciplinary and cross-sectoral approach is necessary (Bidaisee & Macpherson, 2014; Cerdà-Cuéllar et al., 2019; Kahn et al., 2016).

6. Conclusions

An insight into the molecular epidemiology of *Campylobacter lari* in skuas from the Southern Ocean was performed using flaA-RFLP and PFGE genotyping techniques. Both proved to be very useful to study the genetic diversity of the isolates, being PFGE the one with a stronger discriminatory level.

A high number of molecular profiles were found, that evidence the genetic diversity of *C. lari* in skuas. The similarities of some pulsotypes between the different species of skuas and sampling sites are consistent with the distribution of this migratory birds and the overlap of hybridization sites during breeding season. The role of migration and hybridization between brown and south polar skuas posed as the reason of the interconnectivity of the islands

The role of wild birds is key to a better understanding of the transmission of pathogens in the Antarctic, the introduction of emergent diseases and its potential impact and relation to human health.

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