Simultaneous analysis of the intestinal parasites and diet through eDNA metabarcoding

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Abstract

Agriculture expansion and intensification are having a huge impact on plant and arthropod diversity and abundance, affecting food availability for farmland birds. Difficult food access, in turn, can lead to immunosuppression and a higher incidence of parasites. In studies designed to examine changes in the diet of birds and their parasites, metabarcoding is proving particularly useful. This technique requires mini-barcodes capable of amplifying the DNA of target organisms from faecal eDNA. To help understand the impact of agricultural expansion, this study sought to design and identify mini-barcodes that might simultaneously assess diet and intestinal parasites from the faeces of farmland birds. The capacity to identify diet and parasites of two existing and three developed mini-barcodes was tested "in silico" in relation to the behaviour of a reference eukaryotic primer set. Of the mini-barcodes tested, MiniB18S_81, one of our designs, showed the higher taxonomic coverage of eukaryotic taxa and a greater amplification and identification capacity for diet and parasite taxa sequences. This primer pair was tested on faeces samples from five different steppe bird species. According to our data, this barcode shows good taxonomic resolution of the most relevant diet and parasite phyla, Arthropoda, Nematoda, Platyhelminthes and Apicomplexa, at the order level. The mini-barcode developed emerges as an excellent tool to simultaneously provide detailed information regarding the diet and parasitology of birds, essential for conservation and management.

Introduction

Owing to ever-changing climate and land use patterns, biodiversity is increasingly under threat (Pimm et al., 1995; Jetz et al., 2007). In the last 50 years, especially, agricultural expansion has brought about changes in land use that have severely compromised biodiversity (Meyer & Turner, 1992; Matson et al., 1997; Pain & Pienkowski, 1997; Newton, 1998; Chamberlain et al., 2000; Benton et al., 2003). Among other consequences, expanding croplands are diminishing plant species abundance and richness (Wilson et at., 1999; Storkey et al., 2012; Fonderflick et al., 2020), and consequently seed banks (Wilson et at., 1999; Andreasen et al., 2018) and the seasonality of seed availability (Newton, 2004). Further, as the seeds used in intensive agriculture are mostly treated, this also has devastating repercussions on the birds that ingest these seeds (Prosser & Hart, 2005; Lopez-Antia et al., 2015; Millot et al., 2017; Eng et al., 2019). The intensification of agriculture has been also identified as the main driver of declining insect populations (Benton et al., 2002), which are also a dietary component of many bird species. This decline in insect populations is already having a notable impact on insectivorous bird communities (Bowler et al., 2019). Land use changes and reduced food availability are a stress factor for birds, affecting their health and immune response (Kitaysky et al., 1999; Hoi-Leitner et al., 2001; Kitaysky et al., 2007; Pigeon et al., 2013; Almasi et al., 2015) also lowering their defences against parasites (Korschgen et al., 1978; Wakelin, 1996; Nordling et al., 1998). In effect, synergistic effects of food availability, parasitism and stress have been observed on population densities (Chapman et al., 2006). Disease/parasitism and nutrition often interact to determine the abundance of wildlife populations. Helminth and protozoan parasites can impact host survival and reproduction directly through pathological

effects and indirectly by compromising the host's health state (Coop & Holmes, 1996; Murray et al., 1998). Hence, parasitism is also an important factor to consider as a potential risk for both wild and farm-land bird species (Lafferty, 1997; Dunn et al., 2014; Stockdale et al., 2015; Cabodevilla et al., 2020).

To date, studies designed to address the diet of farmland birds have paid most attention to available plants and arthropods, without confirming whether these were consumed or not (Salamolard & Moreau, 1999; Holland et al., 2006; Faria et al, 2012; Holland et al., 2012). Other studies focusing more on the prey ingested have examined this issue through visual identification of the remains of prey exoskeletons present in the faeces of these birds (Jiguet, 2002; Browne et al., 2006; Holland et al., 2006; Bravo et al., 2017). This identification method, widely used for diet analysis, does not generally go beyond the ordinal taxonomic level and few individuals are identified at deeper levels (Jiguet, 2002; Browne et al., 2006; Bravo et al., 2017). In addition, some prey remains could go undetected or unidentified because of difficulties in identifying diet components after they have been digested (Moreby, 1988; Pompanon et al., 2012). The situation for parasitological studies conducted in farmland birds is similar as most have involved the visual identification of parasites (Browne et al., 2006; Okulewicz & Sitko, 2012; Rengifo-Herrera et al., 2014; Presswell & Lagrue, 2016) often via the dissection of dead animals (Villanúa et al., 2008; Santoro et al., 2010; Okulewicz & Sitko, 2012). However, in studies based on faeces samples (non-invasive), the most abundant parasites can be visually identified but low intensity parasitism is hardly discernible (Rengifo-Herrera et al., 2014; Presswell & Lagrue, 2016). Moreover, taxonomic identification based only on the morphological appearance of the eggs of many species is also limited or even impossible (Browne et al., 2006; Presswell & Lagrue, 2016).

The introduction twenty years ago of environmental DNA analysis opened a new avenue for ecology science (Taberlet et al., 2012b). This method has quickly gained importance in parallel with new-generation sequencing technology (Shokralla et al., 2012; Taberlet et al., 2012a, b) and is becoming increasingly popular for the study of diet, microbiology and parasitology (Pompanon et al., 2012; Shokralla et al., 2012; Taberlet et al., 2012a; Bass et al., 2015; Kerley et al., 2018). Through eDNA analysis, useful information can be obtained from non-invasive samples such as faeces (Srivathsan et al., 2016), thus avoiding the need to examine dead animals. Besides parasites, faeces samples can also provide useful information on host genetics, gut microbiota and diet (Srivathsan et al., 2016). However, faeces usually contain various substances that act as PCR inhibitors (Lantz, 1997; Wilson, 1997; Radstrom et al., 2004) and faecal DNA is also fairly degraded (Deagle et al., 2006). This determines a need to design proper primers for eDNA analysis.

To date, many different barcodes, both specific and broad-spectrum primers, have been designed for DNA metabarcoding (Prosser et al., 2013; Hadziavdic et al., 2014; Van Steenkiste et al., 2015; Cheng et al., 2016; Krehenwinkel et al., 2018). However, the large size of the DNA fragments targeted sometimes makes these primers impractical for environmental metabarcoding studies. In effect, DNA from water, soil, air or faeces tends to be quite degraded (Deagle et al., 2006; Yu et al., 2012; Taberlet et al., 2012a) so there is a need to be careful with the length of the barcode used (Hajibabaei et al., 2006; Deagle et al., 2006; Deagle et al., 2007; Taberlet et al., 2012b). Studies that have focused on the metabarcoding of eukaryotic eDNA have already described specific mini-barcodes for different taxa (Epp et al., 2012; Pompanon et al., 2012). However, using these specific mini-barcodes, analysis focuses only on the target taxa, and no information is provided on the importance of these taxa in the sample. In contrast, broad-spectrum primers (usually with lower resolution) do provide information on both target taxa and on their overall contribution to a higher category taxon. For example, a eukaryotic broad-spectrum primer pair used on faeces samples could simultaneously provide information on host genetics, diet and intestinal parasites. However, as far as we know, the eukaryotic mini-barcodes available have not been tested with this purpose in mind. We propose that broad-spectrum primers could be useful tools for comparative studies. While, so far, metabarcoding cannot be considered a quantitative tool, several studies have shown some quantitative capacity of this method (Evans et al., 2016; Lamb et al., 2019; Pinol et al., 2019). Accordingly, it should be possible to compare the proportions of each taxon among similar samples, e.g., water samples from different ponds of faeces collected in different seasons (Pompanon et al., 2012). The use of candidate primers to identify taxa, nevertheless, depends on the existence of reference sequence data (Clarke et al., 2014) so broad-spectrum barcodes need a robust reference dataset. Moreover, for an accurate estimate of biodiversity, a sufficiently variable DNA region needs to be

amplified. The most used DNA genes for barcode design are COI, cytb, 12S, 16S, 18S, ITS1, ITS2 and rbcL (Hajibabaei et al., 2007; Pompanon et al., 2012; Andujar et al., 2018; Djurhuus et al., 2020). Among these, the 18S rRNA gene of the small ribosomal subunit (SSU) spans an especially variable region for which there exists a robust and constantly expanding reference dataset (Hadziavdic et al., 2014).

The present study was designed to identify a mini-barcode for use in metabarcoding that can provide information from faeces samples on both the diet and intestinal parasites of birds. We assessed existing primers as well as self-designed primers targeting the 18S rRNA gene in terms of their suitability for use on bird faeces samples. Once we had identified a suitable mini-barcode, it was also tested empirically using a high throughput sequencing approach on faeces samples of different steppe bird species.

Methods

Primer design

Our main goal was to cover the broadest possible spectrum of main phyla present in the diet and of parasites of birds. We used a dataset of 5000 sequences selected randomly from SILVA SSU v132 reference and non-redundant dataset (Quast et al., 2013), downloaded via the function *obisilva* of Obitools software (Boyer et al., 2016). The selection process was based on sequence annotation of the Silva database including 1000 sequences for each phylum Phragmoplastophyta, Apicomplexa, Arthropoda, Nematoda and Platyhelminthes. These were aligned in Geneious v11.1.4 (https://www.geneious.com; Kearse et al., 2012) and the resulting alignment formed the basis for the design of the primers. Primer design was performed using Geneious v11.1.4. (Biomatters Ltd, New Zealand) with the following parameters: primer length 18–27 nucleotides, melting temperature (Tm) 57–63degC and GC content 30–80%. Next, we assessed primer pairs for their potential to form dimers or alternative amplicons. Two approaches were implemented: 1) 100 mini-barcodes (amplicon size $<$ 200 bp) were designed to cover the sequences of Nematoda and Platyhelminthes phyla (2000 sequences; Supplemental information: Table S1); and 2) 20 mini-barcodes (amplicon size 100-200 bp) were designed using the full dataset of 5000 sequences (Supplemental information: Table S2). Mini-barcodes were then selected according to the following criteria.

1. Barcodes appearing in both approaches.
2. Barcodes covering a variable region (V1 to V9) of the 18S rRNA gene.
3. Barcodes for which primers were designed to cover the full alignment area. Primers covering the edge of the alignment area were discarded as in this region there was no information for many sequences.
4. In the case of barcodes generated with similar primers, we selected the longer mini-barcode.

After this filtering, three mini-barcodes were selected, two (MiniB18S_41 and MiniB18S_43) for the V3 region and one (MiniB18S_81) for the V7-V8 region (Table 1).

Primer test on real tissue

We first tested whether the designed primers (Table 1) could amplify DNA in simple PCRs conducted on 7 tissue samples of 6 different phyla: a nematode (*Ascaridia galli*), cestode (*Raillietina cesticillus*), insect (*Apis mellifera*), arachnid (*Ischyropsalis pyrenaea*), leech (*Helobdella stagnalis*), gastropod (*Cepaea hortensis*) and bird (*Scolopax rusticola*). DNA extraction from tissues was performed using the kit DNeasy(r) Blood & Tissue Kit (Ref. 69506) (Quiagen). All PCR reactions (20 µl) were carried out using 1 µl of DNA in reactions with 1.25 µl of MgCl (25 mM), 2 µl of dNTPs (20 mM), 0.25 of 10X Buffer B, 0.3 µl of each forward (20 pM) and reverse (20 pM) primers, 1 µl of BSA (10mg/ml) and 0.25 µl of BIOTAQ DNA Polymerase of Bioline (ref. BIO-21060; 5 U/µl). The thermocycle conditions were: 96°C for 1 minute and 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 1 minute. PCR amplification capacity was confirmed by electrophoresis in 1.5% TBE agarose gel.

"In silico" PCR

We then went on to check by "in silico" PCR the new mini-barcodes (Table 1) and two eukaryotic mini-barcodes of the 28S rRNA gene, as the mini-barcodes most widely used in next generation sequen-

cing (NGS) diet studies (Pompanon et al., 2012): nucLSUDf1/nucLSUDr1 (Sonnenbe et al., 2007) and Short28SF/Short28SR (Vestheim & Jarman, 2008). In addition, we compared the taxonomic coverage and resolution of the mini-barcodes to those of a reference eukaryotic primer set F566/R1200 (Hadziavdic et al., 2014) (Table 1). This F566/R1200 barcode has been identified as the best eukaryotic barcode among "de novo" designed and previously existing universal eukaryotic 18S rRNA barcodes (Hadziavdic et al., 2014).

All primers were "in silico" tested using the program ecoPCR (Ficetola et al., 2010) and Obitools (Boyer et al., 2016). First we used the*obisilva* function to download the ecoPCR format SILVA 132 SSU reference (cleaned to keep only high quality sequences) and non-redundant dataset. Likewise, we downloaded the ecoPCR format SILVA 132 LSU reference dataset. For large subunits (LSU), SILVA has no non-redundant version. Second, we used the function *ecotaxstat* of ecoPCR (Ficetola et al., 2010) to test the taxonomic coverage of the 3 designed mini-barcodes, F566/R1200 eukaryotic long barcode (650bp), and nucLSUDf1/nucLSUDr1 and Short28SF/Short28SR 28S mini-barcodes on Bacteria, Archaea and Eukarya. In the same way, we used the functions*ecotaxstat* and *ecotaxspecificity* to test their taxonomic coverage and resolution capacity on 10 eukaryotic phyla (Supplemental information Table S3): Apicomplexa, Nematoda and Platyhelminthes including main parasites of birds; Phragmoplastophyta, Annelida, Arthropoda and Mollusca which may appear in birds' diets; Ascomycota and Basidiomycota to check for fungal sample contamination; and Vertebrata to check if the barcodes could identify the host. Figure 3 focuses on the phyla Phragmoplastophyta, Apicomplexa, Arthropoda, Nematoda and Platyhelminthes, as we anticipated these would be the taxa mostly represented in the diet and parasites of birds. The ecotaxspecificity option *-e 0* was used, which does not allow any mismatches. Taxon classification was according to Silva's reference database (www.arb-silva.de).

Metabarcoding proof

Finally, based on taxonomic amplification and resolution capacities revealed in the "in silico" PCRs, we selected the MiniB18S_81 mini-barcode for assessment in a real metabarcoding analysis. The samples tested were faeces samples collected (in Spain) from 12 bird flocks of 5 different steppe bird species; 1 flock of farmed red-legged partridges (*Alectoris rufa*), 2 flocks of wild red-legged partridges (*Alectoris rufa*), 4 flocks of pin-tailed sandgrouse (*Pterocles alchata*), 1 flock of black-bellied sandgrouse (*Pterocles orientalis*), 2 flocks of great bustards (*Otis tarda*) and 2 flocks of little bustards (*Tetrax tetrax*).

Whenever possible, fresh faeces from 20 individuals in each flock were collected. Faeces were collected in the roosts without trapping any birds. DNA extractions were performed using the QIAamp® DNA Stool Mini Kit from Quiagen (Ref. 51504). From 15 to 20 DNA samples were obtained per flock (DNA could not be extracted from every sample). The concentration (µg/ml) and quality of each DNA sample were measured using the NanoVue Plus spectrophotometer (GE Healthcare). Samples from each flock were mixed according to DNA quantity and quality in 3 pools of 5-7 samples, one of high, one of medium and one of low quantity. Subsequently, DNA was PCR amplified from extracts using each of the designed primers (miniB18S_81F and miniB18S_81R) (Table 1) at the Analytical Services (SGIker) of the University of the Basque Country, UPV/EHU. To retrieve enough material for the sequencing reaction, three PCR reactions were run per sample pool. Samples were purified and a second reaction was performed to index each amplified product and attach Illumina adaptors using the Illumina Nextera v2 kit. We used the same Illumina adaptor for the three pools prepared for each flock. Amplification (20 µl) was carried out using 1 µl of DNA in a reaction mix containing 1.25 µl of MgCl (25 mM), 2 µl of dNTPs (20 mM), 0.25 of 10X Buffer B, 0.3 µl of each forward (20 pM) and reverse (20 pM) primer, 1 µl of BSA (10mg/ml) and 0.25 µl of GoTaq® Flexi DNA Polymerase from Promega (ref. M829; 5 U/µl). The thermocycle conditions were: 96°C for 1 minute and 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 1 minute. Once amplified, PCR outputs were sequenced in an Illumina MiSeq NGS platform (sequencing of 2x150 bp paired-end reads) with the MiSeq Reagent Kit v2, following the manufacturer's instructions.

Bioinformatic sequencing output tests were conducted using the Cutadapt (Martin, 2011) and Usearch (Edgar, 2010) software packages. First, the adapters were cut and the forward and reverse sequences combined. The sequences were then cleaned and OUT tables built based on 97% OTUs. We used the *join.fastq* function of Usearch instead of merging forward and reverse sequences to avoid losing sequences of amplicons longer

than 300 bp. Subsequently, the *sintax* function of Usearch was used to predict the taxonomy of the OTUs obtained with a*cutoff* of *0.8* and a combined 18S and 16S dataset. The reference dataset was built combining the Silva 18S v123 dataset (Quast et al., 2013) with the RDP 16S v16 training set (Cole et al., 2014), both provided by the Usearch platform. We opted for the RDP 16S v16 training dataset instead of a complete Silva, Greengenes or RDP 16S dataset because of the high level of mistaken sequences reported for these datasets (Edgar, 2018).

All graphs were constructed using the *ggplot2* package (Wickham 2016) in R v3.6.1 (R Core Team 2019).

Results

"In silico" testing

Before this test, it was verified through real-time PCR that the three new mini-barcodes showed the capacity to amplify the DNA of the 7 different tissue samples belonging to 6 different phyla.

In the "in silico" PCR, all the barcodes tested showed high taxonomic coverage of eukaryotic organisms (Figure 1), along with a null capacity to amplify organisms of the taxa Bacteria and Archaea (Figure 1). Of the three mini-barcodes designed in this study, MiniB18S_81 showed the higher taxonomic coverage of eukaryotic organisms. This barcode served to amplify 91.1% of eukaryotic sequences present in the SILVA reference database, which is more than the other 28S mini-barcodes tested in this study. Only the large barcode 566F/1200R showed a slightly better amplification capacity for eukaryotic organisms (92.6%; Figure 1). In addition, all barcodes showed a good capacity to amplify eukaryotic phyla sequences: MiniB18S_41 = 94.9% (131/138); MiniB18S_43 = 93.5% (129/138); MiniB18S_81 = 95.7% (132/138); 566F/1200R = 98.6% (136/138); nucLSUDf1/nucLSUDr1 = 96.6% (85/88); and Short28SF/Short28SR = 96.6% (85/88).

The six barcodes were able to amplify large numbers of organisms of the 10 target phyla (Supplemental information: Table S3). However, MiniB18S_81 amplified more than 90% of the sequences of 7 of these 10 phyla, and showed the best taxonomic coverage among the mini-barcodes tested, as good as the amplification capacity of the large barcode 566F/1200R (Supplemental information: Table S2). Of the previously designed 28S mini-barcodes, nucLSUDf1/nucLSUDr1 showed poor taxonomic coverage of several phyla, amplifying less than 80% of the sequences of 5 of the 10 phyla examined (Supplemental information: Table S3).

MiniB18S_43 and the reference nucLSUDf1/nucLSUDr1 mini-barcodes showed the worst capacity to detect sequences of the phyla Phragmoplastophyta, Arthropoda, Nematoda, Platyhelminthes and Apicomplexa (Figure 2). The former did not feature a high amplification capacity for Nematodes and the latter behaved poorly when used on all taxa, especially Nematoda and Apicomplexa (Figure 2). The MiniB18S_81 mini-barcode provided the best results, showing an excellent amplification capacity on Arthropoda, Nematoda, Platyhelminthes, despite a slightly lower amplification capacity for the Apicomplexa phylum than MiniB18S_41, MiniB18S_43 and Short28SF/Short28SR (Figure 2). It should also be mentioned that all five mini-barcodes and 566F/1200R displayed a high amplification capacity for the Fungi kingdom and its phyla Ascomycota and Basidiomycota (Supplemental information: Table S3).

All tested barcodes showed short mean amplicon lengths (Table 1), as well as a quite constant amplicon length (Supplemental information: Figure S1). Although the five mini-barcodes generated amplicons of 30 to 900 bp, percentages of sequences longer than 300 bp were reduced (0.06% MiniB18S_41; 0.05% MiniB18S_43; 1.16% MiniB18S_81, 0.34% nucLSUDf1/nucLSUDr1 and 0.32% Short28SF/Short28SR). In the case of MiniB18S_81, cestodes showed larger amplicons with a mean length of 299 bp including some longer than 300 bp.

In contrast, our taxonomic resolution revealed a higher resolution capacity of the large barcode 566F/1200R than any of the mini-barcodes (Table 2; Supplemental information: Table S3). The resolution capacity of the five mini-barcodes was not poor (Supplemental information: Table S3), yet MiniB18S_81 seemed to have the best resolution capacity for the phyla Phragmoplastophyta, Arthropoda, Nematoda, Platyhelminthes and Apicomplexa (Table 2). In effect, this mini-barcode offered high taxonomic resolution at the order level within the phyla Arthropoda, Nematoda, Platyhelminthes and Apicomplexa, at the family level in Platyhelminthes

and Apicomplexa, at the genus level in Apicomplexa, and at the species level in Arthropoda, Nematoda and Platyhelminthes (Table 2). However, no results were obtained at the family and genus levels for various phyla as this information was lacking in the reference dataset (Table 2).

Metabarcoding proof

The MiniB18S_81 mini-barcode provided high quality sequences. On average, 66% of forward/reverse sequences combined showed less than one expected error. Moreover, on average, 80.4% of reads were assigned to an OTU with 97% identity. Taking as a reference dataset the Silva 18S v123 dataset and RDP 16S v16 training dataset combined, the MiniB18S_81 mini-barcode served to identify 1367 of 1384 OTUs recovered as eukaryotic organisms with certainty, whereas no Bacteria or Archaea were thus identified.

Our metabarcoding analysis results were also consistent with the data obtained through "in silico" PCR. As "in silico" (Table 2), the MiniB18S_81 mini-barcode showed an excellent taxonomic resolution capacity at the order level for Apicomplexa, Arthropoda, Nematoda and Platyhelminthes, while for plants it only showed good resolution at the class level (Table 3). In the Apicomplexa phylum, this mini-barcode also offered good resolution capacity at the family and genus levels (Table 3). However, no results were obtained at the family and genus levels for various phyla, due to the lack of information in the reference dataset regarding these taxonomic levels and phyla (Table 3). We did not include the Annelida and Mollusca phyla in Table 3 as only two OTUs of each were detected in this analysis. Nevertheless, this mini-barcode also showed good taxonomic resolution at the class level in Ascomycota and Basidiomycota (Table 3). In contrast, its taxonomic resolution of the Vertebrata phylum was poor, as expected considering the results of the "in silico" approach. In addition, this barcode was capable of unambiguously identifying some genera. Among these, we should highlight *Cryptosporidium* spp. and *Blastocystis* spp., as two groups of organisms generating much interest because of their impacts on human health.

In addition, the use of this mini-barcode rendered a percentage of reads of each taxon in relation to the total reads obtained for each sample (Figure 3). We were also able to estimate the percentage of reads of a specific taxon associated with diet in relation to the total reads of the taxon component of the diet, or the percentage of reads of one protozoan parasite in relation to the total reads of protozoans in the gut microbiota. This type of information gives an idea of the contribution of each taxon to the gut microbiota, diet or parasite community.

Discussion

The aim of this study was to find the best possible set of eukaryote 18S rRNA primers to be used in studies based on faeces metagenomics for the simultaneous analysis of diet and parasites in threatened farmland birds. The mini-barcodes developed in this study were found to meet these expectations. The primer set MiniB18S_81F/MiniB18S_81R in particular showed great taxonomic coverage of almost all eukaryotic taxa, as well as good taxonomic resolution of the most interesting phyla associated with the diet and parasitology of birds both through both "in silico" and real metabarcoding testing.

According to our results, of the three new mini-barcodes, MiniB18S_81 was by far the best although the other two mini-barcodes designed, MiniB18S_41 and MiniB18S_43, did not show poor behaviour. Both achieved quite high taxonomic coverage of Apicomplexa, Arthropoda, Chloroplastica, Nematoda and Platyhelminthes, but also displayed a lower taxonomic coverage and resolution than MiniB18S_81. This was nevertheless expected as the V7-V8 region of the 18S gene amplified by MiniB18S_81 is much more variable than the V3 region amplified by MiniB18S_41 and MiniB18S_43 (Hadziavdic et al., 2014). Moreover, the MiniB18S_81 mini-barcode showed a much wider amplification capacity than the 28S mini-barcode nucLSUDf1/nucLSUDr1 (Sonnenbe et al., 2007) and proved slightly superior to the Short28SF/Short28SR (Vestheim & Jarman, 2008) 28S mini barcode for four of the five target phyla. MiniB18S_81 displayed especially good amplification skills on Arthropoda, Nematoda and Platyhelminthes. In addition, it showed a great taxonomic resolution capacity at the order level in four of five target phyla in the metabarcoding test conducted on bird faeces. Its good taxonomic resolution at the arthropod order level should also be mentioned. Around 83.5% of arthropod OTUs obtained in the metabarcoding test were unambiguously identified. Hence this mini-barcode could

provide information on the contribution to the bird's diet of different arthropod orders, other invertebrates and plants. Although its taxonomic resolution within plants was poor, it did provide an overall picture of the importance of plants in the diet. According to our "in silico" and metabarcoding tests, the mini-barcode proposed here allowed for the accurate identification of the orders of most Apicomplexa, Nematoda and Platyhelminthes organisms (using SILVA SSU v132 dataset). In fact, this barcode could provide detailed information useful for comparative parasite load studies. The lower taxonomic resolution observed at species level in the metabarcoding test is likely attributable to the fact that many of the OTUs recovered corresponded to organisms that were not included in the reference dataset. Thus according to our "in silico PCR" analysis, MiniB18S_81 shows good taxonomic resolution at the species level of Arthropoda, Nematoda and Platyhelminthes using a suitable reference database. Although, the percentage of reads obtained for each taxa cannot be considered quantitative of the real contribution of these taxa to the gut microbiota (Evans et al., 2016; Lamb et al., 2019; Piñol et al., 2019), this variable could be useful for comparative studies.

The capacity of MiniB18S_81 to unambiguously identify some pathogens such as the genera *Cryptosporidium* and *Blastocystis*suggests its possible use for estimating parasite loads. In effect, there is much interest in *Blastocystis* spp. because of their impacts on human health (Tan, 2008; Scanlan, 2012) and zoonotic capacity (Greige et al., 2018). Studies of *Blastocystis* spp. have been often based on presence/absence data and this new mini-barcode could provide information on the proportions of this organism in the host's gut microbiota especially useful for comparative studies.

All the tested barcodes showed quite short but constant amplicon lengths. While amplicon lengths ranged from 30 to 900 bp for all the studied primers, few were longer than 300 bp. In the case of MiniB18S_81, one of the taxa eliciting amplicons longer than 300 bp was Cestoda, with a mean of 299 bp. This is because Cestoda have an insertion of more than 100 bp in this region. However, this should not be a problem as Cestoda DNA in the samples is not degraded as it is tissue DNA (adults, larvae or eggs).

All barcodes displayed really good coverage of the Fungi kingdom and its phyla Ascomycota and Basidiomycota. This is important as, when working with eDNA, environmental contamination particularly with fungi could be a problem (Bohmann et al., 2014). When animal faeces is sampled from the ground, the time interval between deposition and sampling should be minimal, as a longer interval will mean a greater likelihood of sample colonization by other organisms such as fungi or bacteria. Further, target organisms can also die due to the aerobic conditions and be degraded by bacteria and fungi. Hence, it is essential to collect fresh faeces. Using the broad-spectrum barcodes proposed here, fungal contamination can be identified and contaminated samples discarded.

All things considered, the barcodes tested featured quite good qualities. The Short28SF/Short28SR primer pair designed by Vestheim and Jarman (2008) showed wide taxonomic coverage of all the phyla examined along with an acceptable and constant taxonomic resolution capacity within these phyla. The only drawback of this mini-barcode is that the available reference datasets for LSU are not as long and purified as those for SSU, and without a robust reference database, information is lost. The MiniB18S_81 mini-barcode was also very constant, but was better at detecting the most important diet and parasite phyla than Short28SF/Short28SR, as it showed a high taxonomic resolution capacity for these phyla. In contrast, nucLSUDf1/nucLSUDr1 (Sonnenbe et al., 2007) did not behave adequately for the present purpose. This mini-barcode amplified less than 90% of sequences belonging to 8 of 10 studied phyla, which is far from the values shown by the other primer pairs. Although its taxonomic resolution capacity was also high, its taxonomic coverage (82% of eukaryotic organisms) was the lowest of all the primers tested. We would thus recommend the use of MiniB18S_81 for faecal metagenomics studies. For other target taxa, primers should be first tested for their suitability.

For ecological studies and biomonitoring programmes, high throughput sequencing holds great promise. The optimal primers proposed here were able to generate amplicon sequences with good resolution when applied to bird faeces samples and thus seem exceptional candidates for gut and faeces eDNA metabarcoding. Useful information was obtained about the arthropod orders and plants in the diet. An idea was also provided of the contributions of Nematoda, Platyhelminthes and Apicomplexa parasites to the gut microbiota. MiniB18S_81

could thus be a new tool for simultaneous biomonitoring of diet and intestinal parasites through eDNA metabarcoding of bird faeces samples. Understanding the ecological determinants of animal density is a central question in the field of species conservation, especially if we consider the current rate of human interference. It is in this context that MiniB18S_81 could have important implications offering more and better information on the ecology of farmland birds and on the reasons for their decline.

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Data Accessibility

The data that support the findings of this study are openly available in Dryad at XXXX, reference number XXXX.

Author contributions

M.J.M. designed the study and mini-barcodes. X.C. collected the samples, performed the lab work, bioinformatics analyses and wrote the first draft of the manuscript. M.J.M, B.J.G-M. and X.C. prepared the manuscript and contributed to subsequent drafts.

Tables

Mini-barcode	Primer	Mean length	Reference
MiniB18S_41	**F**: CAGGGTTCGATTCCGGAGAG **R**: CACCAGACTTGCCCTCCAAT	156 bp	This study
MiniB18S_43	**F**: AACGGCTACCA-CATCCAAGG **R**: CACCAGACTTGCCCTCCAAT	125 bp	This study
MiniB18S_81	**F**: GGCCGTTCT-TAGTTGGTGGA **R**: CCCGGACATCTAAGGGCATC	150 bp	This study
566F/1200R	**566F**: CAGCAGCCGCGGTAATTCC **1200R**: CCCGTGTTGAGTCAAATTAAGC	610 bp	Hadziavdic et al., 2014
nucLSUDf1/nucLSUDr1	**nucLSUDf1**: CGTCTTGAAACACGGACCAAG **nucLSUDr1**: GCATAGTTCACCATCTTTCGGG	156 bp	Sonnenbe et al., 2007
Short28SF/ Short28SR	**Short28SF**: GTGTAACAACTCACCTGCCG **Short28SR**: GCTACTACCACCAAGATCTG	154 bp	Vestheim & Jarman, 2008

Table 1. List of barcodes tested in this study. More information on amplicon lengths is provided as Supplemental information: Figure S1

Arthropoda	MiniB18S_41	MiniB18S_43	MiniB18S_81	566F/1200R
Class	**71.4%** (10 of 14)	**69.2%** (9 of 13)	**76.9%** (10 of 13)	**85.7%** (12 of 14)
Order	**52.6%** (40 of 76)	**50%** (38 of 76)	**73%** (54 of 74)	**89.5%** (68 of 76)
Species	**21.1%** (2623 of 12445)	**19.5%** (2434 of 12489)	**59.2%** (7451 of 12591)	**76.8%** (9752 of 127
Phragmoplastophyta	**MiniB18S_41**	**MiniB18S_43**	**MiniB18S_81**	**566F/1200R**
Class	**33.3%** (1 of 3)	**33.3%** (1 of 3)	**100%** (3 of 3)	**100%** (3 of 3)
Order	**8.6%** (10 of 117)	**8.6%** (10 of 117)	**31.2%** (37 of 119)	**80.3%** (94 of 117)
Genus	**18.6%** (336 of 1804)	**17.0%** (307 of 1808)	**39.4%** (706 of 1791)	**87.3%** (1531 of 175
Species	**29.4%** (2265 of 7709)	**28.3%** (2254 of 7975)	**34.5%** (2676 of 7756)	**65.9%** (5013 of 760
Nematoda	**MiniB18S_41**	**MiniB18S_43**	**MiniB18S_81**	**566F/1200R**
Class	**100%** (2 of 2)	**100%** (2 of 2)	**100%** (2 of 2)	**0%** (0 of 2)
Order	**26.67%** (4 of 15)	**20%** (3 of 15)	**53.3%** (8 of 15)	**80%** (12 of 15)

Arthropoda	**MiniB18S_41**	**MiniB18S_43**	**MiniB18S_81**	**566F/1200R**
Species	**47.5%** (932 of 1961)	**46.6%** (727 of 1560)	**61.6%** (1270 of 2062)	**86.4%** (1811 of 209
Platyhelminthes	**MiniB18S_41**	**MiniB18S_43**	**MiniB18S_81**	**566F/1200R**
Class	**100%** (6 of 6)	**100%** (6 of 6)	**100%** (6 of 6)	**100%** (6 of 6)
Order	**79.5%** (31 of 39)	**71%** (27 of 38)	**89.7%** (35 of 39)	**94.9%** (37 of 39)
Family	**86.11%** (31 of 36)	**83.8%** (31 of 37)	**100%** (37 of 37)	**100%** (37 of 37)
Species	**52.2%** (900 of 1723)	**49.3%** (837 of 1699)	**81.5%** (1523 of 1869)	**92.9%** (1567 of 168
Apicomplexa	**MiniB18S_41**	**MiniB18S_43**	**MiniB18S_81**	**566F/1200R**
Class	**100%** (4 of 4)	**100%** (4 of 4)	**100%** (4 of 4)	**100%** (4 of 4)
Order	**100%** (5 of 5)	**100%** (5 of 5)	**100%** (5 of 5)	**100%** (5 of 5)
Family	**100%** (5 of 5)	**100%** (5 of 5)	**100%** (5 of 5)	**100%** (5 of 5)
Genus	**76.2%** (48 of 63)	**73.8%** (45 of 61)	**70.97%** (44 of 62)	**96.9%** (63 of 65)
Species	**34.03%** (487 of 1431)	**31.6%** (453 of 1435)	**41.3%** (526 of 1274)	**76%** (1122 of 1477)

Table 2. "In silico" resolution capacity of barcodes for the phyla Phragmoplastophyta, Arthropoda, Nematoda, Platyhelminthes and Apicomplexa. Resolution percentages shown only for the taxonomy categories for which there were sufficient data available. To see all the taxonomic resolution results go to Supplemental information: Table S3

MiniB18S_81	**MiniB18S_81**	**Taxonomic resolution**	**Taxonomic resolution**	**Taxonomic resolution**
Kingdom	**Phylum**	**Phylum**	**Class**	**Order**
Alveolata		82.9%	75%	71.1%
	Apicomplexa		**81.3%**	**81.3%**
Chloroplastida		89.4%	84.8%	7.1%
	Phragmoplastophyta		**93.2%**	**7.7%**
Fungi		84%	59.5%	40.5%
	Ascomycota		67.1%	50.6%
	Basidiomycota		84.6%	48.1%
Metazoa		89.0%	72.0%	63.3%
	Arthropoda		**95.9%**	**84.7%**
	Nematoda		**100%**	**94.4%**
	Platyhelminthes		**91.7%**	**83.3%**
	Vertebrata		17.6%	

Table 3. "In vivo" resolution capacity of the mini-barcode MiniB18S for different taxa including Chloroplastica, Arthropoda, Nematoda, Platyhelminthes and Apicomplexa. Resolution percentages shown only for the taxonomy categories for which there was sufficient taxonomic information in the reference dataset. Annelida and Mollusca excluded due to lack of information.

Figures Legends

Figure 1. "In silico" taxonomic coverage capacity of barcodes of Bacteria, Archaea and Eukarya taxa.

Figure 2. "In silico" taxonomic coverage of barcodes of the phyla Phragmoplastophyta, Arthropoda, Nematoda, Platyhelminthes and Apicomplexa. For all results of taxonomic coverage, go to Supplemental information: Table S3.

Figure 3. Mean percentages of reads of the phyla Phragmoplastophyta, Arthropoda, Nematoda, Platyhelminthes and Apicomplexa by bird species.