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1 # NGS metabarcoding proves successful for quantitative assessment of
2 # symbiont abundance: the case of feather mites on birds

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20
Abstract

21 Understanding the ecological function of species and the structure of communities is crucial in the
22 study of ecological interactions among species. For this purpose, not only the occurrence of particular
23 species but also their abundance in ecological communities is required. However, abundance
24 quantification of species through morphological characters is often difficult or time/money consuming
25 when dealing with elusive or small taxa. Here we tested the use of next-generation sequencing (NGS)
26 for abundance estimation of two species of feather mites (*Proctophyllodes stylifer* and *Pteronyssoides*
27 *parinus*) under five different proportions (16:1, 16:4, 16:16, 16:64, and 16 vs 256 mites) against a
28 mock community composed by *Proctophyllodes clavatus* and *Proctophyllodes sylviae*. In all mixtures,
29 we retrieved sequence reads from all species. We found a strong linear relationship between 454 reads
30 and the real proportion of individuals in the mixture for both focal species. The slope for *Pr. stylifer*
31 was close to one (0.904), and the intercept close to zero (-0.007), thus showing an almost perfect
32 correspondence between real and estimated proportions. The slope for *Pt. parinus* was 0.351 and the
33 intercept 0.307, showing that while the estimated proportion increased linearly relative to real
34 proportions of individuals in the samples, proportions were overestimated at low real proportions and
35 underestimated at larger ones. Additionally, pyrosequencing replicates from each DNA extraction
36 were highly repeatable (R=0.920 and 0.972, respectively), showing that the quantification method is
37 highly consistent given a DNA extract. Our study suggests that NGS is a promising tool for abundance
38 estimation of feather mites' communities in birds.

39

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Keywords

41 COI, high-throughput sequencing, mtDNA, NGS quantitative test, pyrosequencing.

42

43 # Introduction

44 The study of ecological interactions among species requires the understanding of the ecological
45 function of the organisms and the structure of communities. This demands knowing not only the
46 occurrence of species in an area and the intensity of their interactions, but also the quantification of
47 species' abundances (Huber et al. 2007). This is particularly challenging when studying elusive
48 species, degraded material (e.g. diet samples) or when dealing with species difficult to identify by
49 morphology. The latter scenario is common in the study of symbiotic interactions where differentiation
50 of taxa of minute symbionts by morphological characters is often complicated. Species-level
51 identification is the most challenging task (Carew et al. 2013), producing more errors than higher
52 taxonomical level identification (Jones 2008), and commonly only some sex and/or age classes (e.g.
53 adult males) allow morphological identification to the species level. This, jointly with the typical large
54 numbers of symbionts (hundreds or thousands in a single host), makes the quantification of the
55 abundance of each individual symbiont at the species level highly challenging when not simply
56 impossible.

57 As a result, molecular techniques based on DNA sequencing have been developed as an
58 alternative to morphological identification, because their potential to obtain fast, easy and accurate
59 identification to the species level. Hebert *et al.* (2003) promoted the use of cytocrome oxidase I (COI)
60 as a molecular barcode for identifying animal species, which have been shown to be successful for
61 most invertebrates (with few exceptions, e.g. Meier et al. 2006; McFadden et al. 2011). Furthermore
62 mtDNA is easy to amplify due to the high number of copies present in cells (Galtier et al. 2009).

63 Development of Next-Generation Sequencing (NGS) technologies has allowed the
64 characterization of communities through the assessment of richness indices of species through DNA
65 barcodes. This is commonly known as "metabarcoding" which refers to identification of multiple
66 species appearing in a bulk sample or degraded material (Taberlet et al. 2012). Several studies have
67 shown the utility of NGS for diversity assessment in: microbial flora of humans (Ahn et al. 2011;
68 Marzorati et al. 2013), and of comestibles (Nam et al. 2012), diet studies (Soininen et al. 2009; Deagle

69 et al. 2009) and natural environmental samples (Yu et al. 2012; Carew et al. 2013; Ji et al. 2013,
70 Gómez-Rodríguez et al. 2015). It is generally assumed that the number of reads obtained from NGS
71 for a given species or taxon positively correlates with its relative abundance in the analysed sample.
72 However, several recent approaches in diverse taxa reveal a more complex scenario. While some
73 controlled tests showed promising results (Hajibabaei et al. 2011; Pilloni et al. 2012; Shokralla et al.
74 2012; Carew et al. 2013; Weber and Pawlowski 2013), other studies concluded that the number of
75 reads of a given taxon was not correlated with its relative abundance (Porazinska et al. 2009; Amend et
76 al. 2010; Egge et al. 2013). This has been proposed to be the consequence of multiple biological and
77 methodological issues (Deagle et al. 2014) such as biases during DNA extraction and PCR
78 amplification (Carew et al. 2013; Kermarrec et al. 2013), assignation of reads to Multiplex Identifiers
79 (Berry et al. 2011), different number of copies of the sequenced gene in different unicellular (Weber
80 and Pawlowski 2013) or multicellular species (e.g. species of different body size, Gómez-Rodríguez et
81 al. 2015), and differential primer affinity for DNA template (Hajibabaei et al. 2011).

82 In this study we experimentally tested the utility of NGS for quantitative assessment of the
83 relative abundance of feather mites (Acariformes: Astigmata: Analgoidea) that live on the surface of
84 wing feathers (Gaud and Atyeo 1996). Although feather mites are the commonest ectosymbionts on
85 birds, their ecology is seldom studied (Dabert and Mironov 1999; Proctor 2003). Their tiny size most <
86 0.5 mm), high number of individuals per host, absence of keys for most genera, low number of expert
87 taxonomists and the fact that for many genera only adult males have clear species-specific morphology
88 make NGS a promising tool for feather mite species identification and relative quantification. Here, we
89 tested whether the number of reads in NGS corresponding to *Proctophyllodes stylifer*
90 (Proctophyllodidae, Buckholz 1869) or *Pteronyssoides parinus* (Pteronyssidae, Koch 1940) correlates
91 with the proportion of individuals of those species in a mock mixture of *Proctophyllodes clavatus*
92 (Fritsch 1961) and *Proctophyllodes sylviae* (Gaud 1957). With this purpose we carried out a controlled
93 test of ten mock communities with five different proportions of a close (same genus) and a distant
94 (different genus) species (Table 1). To look for consistence in pyrosequencing estimates of
95 proportions, we also made five different replicated amplifications of each of the ten DNA templates.

96

Material and methods

Samples collection

Feather mites were sampled from birds captured in two localities in South Spain: Roblehondo (La Iruela), in Sierras de Cazorla, Segura y las Villas Natural Park (Jaén), and Manecorro (El Rocío), in Doñana National Park (Huelva) during August and September 2013. Birds were captured using mist nests, kept individually in cloth bags, immediately banded, inspected for feather mites and released. Four species of feather mites belonging to two genera were collected from the flight feathers (primaries, secondaries and tertiaries) of four different bird host species: *Pr. stylifer* and *Pt. parinus* from blue tits *Cyanistes caeruleus*; *Pr. sylviae* from blackcaps *Sylvia atricapilla* and Sardinian warblers *S. melanocephala*; and *Pr. clavatus* from garden warblers *S. borin*. Given the high specificity of these feather mite species and the low number of mite species found in these birds, feather mite samples could be identified to the species level under the stereomicroscope and stored separately in 98% ethanol until extraction. Later matching of sequences with our DNA barcoding library (Doña et al. 2015) confirmed our initial identifications under the stereomicroscope.

Experimental design

Sets of 16 individuals of *Pr. stylifer* or 16 individuals of *Pt. parinus* were confronted with a varying number of individuals from the mix *Pr. sylviae* / *Pr. clavatus* ("pool" hereafter; Table 1). Only male adult mites were used in this study. In this way, we were confronting the two focal species with different proportions of congeneric (*Proctophyllodes*) or non-congeneric species (*Proctophyllodes* vs *Pteronyssoides*). Once DNA was extracted from each of the ten combinations shown in Table 1, five replicates of each sample were amplified by PCR and pyrosequenced (see below). Thus, we could estimate the correlation between the proportion of individuals introduced in the vials with the proportion of the sequences retrieved, and also the robustness of the pyrosequencing procedure.

DNA extraction

121 Genomic DNA was extracted using HotSHOT (Truett et al. 2000). Total volume of extraction was
122 variable, depending on the number of feather mites being extracted in the same experimental sample.
123 Feather mites in experimental samples with fewer individuals (17-32 mites) were extracted in a final
124 volume of 50 µl and the rest in 100 µl (samples with 80-272 mites). After extraction, exoskeletons
125 were separated from the extraction volume and stored in 98% ethanol.

126 **DNA amplification and sequencing**

127 To build the library of specific amplicons, we used the universal tailed amplification design
128 (Guidelines from amplicon experimental design, ROCHE November 2012). It is based on two rounds
129 of PCR; the first round amplifies the target sequences and the second PCR attaches the multiplex
130 identifiers (MID) and the 454 adaptors to each amplicon. This allowed to barcode and prepare the
131 amplicons for pyrosequencing. In the first PCR, a segment of approximately 650-bp of the COI region
132 was amplified with degenerated primers bcdF05 and bcdR04 (Dabert et al. 2008). PCRs were carried
133 out in 20 µl reaction volumes containing 1x $(NH_4)_2SO_4$ reaction buffer (Bioline), 2.5 mM $MgCl_2$, BSA
134 1x, 0.25 mM dNTPs, 1 µM of each primer, 1.25 U BIOTAQ^{TM} (Bioline) and 5 µl of DNA template.
135 The PCR followed a touchdown PCR profile: initial denaturation for 3 min at 95°C; 20 cycles of
136 denaturing for 1 min at 95°C, annealing for 30 sec starting at 55°C and decreasing by 0.5°C/cycle and
137 elongation for 1 min at 72°C; followed by 20 cycles of denaturing for 1 min at 95°C, annealing for 30
138 sec at 45°C and elongation for 1 min at 72°C; then, a final step for 5 min at 72°C. Five PCR replicates
139 of each extract were done, and thus we carried out 50 PCR reactions in total.

140 PCR products were visualized by electrophoresis in a 2% agarose gel. Visible bands of the
141 COI fragment were cleaned with homemade SPRI beads (Rohland and Reich 2012) and amplified
142 again in the second round. This second PCR was carried out in 20 µl reaction volumes containing 2 µl
143 of 1x $(NH_4)_2SO_4$ reaction buffer (Bioline), 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 1 µM of each primer,
144 1.25 U BIOTAQ^{TM} (Bioline) and 1 µl of PCR product from first PCR, following the profile: initial
145 denaturation for 2 min at 94° followed by 35 cycles of denaturing for 30 sec at 92 °C, annealing for 30
146 sec at 56°C and elongation for 45 sec at 72°C; then, a final step for 5 min at 72°C.

147 The amplification products were electrophoresed in a 2.5% agarose gel and quantitatively
148 assessed using the Quantity One software (Bio-Rad) that estimates band intensities. All PCR products
149 were pooled into equimolar concentrations, purified using SPRI beads and quantified fluorimetrically
150 with Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). The pool was amplified in an emulsion PCR
151 using the GS Junior Titanium emPCR kit (Lib A) with the emPCR amplification protocols for long
152 fragments, and to increase the number of reads we followed the one-way reads amplicon sequencing
153 design (ROCHE Technical Bulletin: Amplicon Sequencing with Various emPCR Amplification
154 Conditions, February 2011; Guidelines from Amplicon Experimental Design, ROCHE November
155 2012). The pool was pyrosequenced on a 454 GS Junior System using the Sequencing Method Manual
156 GS Junior Titanium Series following manufacturer´s instructions (Roche) at Estación Biológica de
157 Doñana (EBD-CSIC) in Seville, Spain.

158 **Bioinformatics analysis**

159 Only high quality fragments of reads between 530 and 850 bp length were used for this experiment.
160 Reads were separated by MID and the Novo Assemble tool of Geneious (Biomatters) was used for
161 collapsing reads within each MID with maximum 3% of mismatches per read. All contigs generated
162 were aligned by MUSCLE with default settings and with known sequences of the species analyzed
163 (Genebank Accession Numbers: *Pr. stylifer*: KP193704 – KP193716; *Pt. parinus*: KP193755 -
164 KP193757; *Pr. clavatus*: KP193516 - KP193523; *Pr. sylviae*: KP193717 - KP193725, Doña et al.
165 2015) . The alignments were visually inspected to identify and count individually the contigs
166 corresponding to each species. In this step, chimeras were identified and omitted. The alignment with
167 all the final sequences used in this experiment is available in Dryad (doi:XXXXXX).

168 **Statistical analyses**

169 Due to the lack of independence of data belonging to the same extraction process, we used Linear
170 Mixed Models (LMMs) to obtain the intercept and slope of the relationship between the proportion of
171 individuals estimated by NGS (dependent variable) and the real proportion of individuals in each
172 extraction for *Pr. stylifer* and *Pt. parinus* (independent variable). The extraction sample was set as the

random effect, and the real proportion of individuals of the focal species in each experimental sample was set as fixed effect.

Results

A total of 84,015 reads were obtained. 49,217 were fragments between 530 and 850 bp of length (corresponding to the COI region). After splitting sequences by MID we obtained a total number of 48,627 reads. 584 reads were not assigned to a known MID or linker, and 6 reads were assigned to a non-present MID. After purging all chimeras and low quality sequences we obtained 36,752 reads corresponding to all 50 MIDs. Median coverage read per MID was 763 (range 18 – 2,545). Only nine of the 50 MIDs had coverage lower than 500 reads per MID of which only four had fewer than 100 reads.

In all 50 mock mixtures, sequences from the focal species (either *Pr. stylifer* or *Pt. parinus*) and from species of the pool were retrieved, even when the species pool was composed of only one or four individuals. Likewise, focal species were always detected even when occurring in the lowest proportions (16 vs 256). There was, however, a likely technical problem with the extraction of DNA from the combination of 16 *Pt. parinus* vs one individual from the pool (Fig. 1A). In any case, the problem occurred before pyrosequencing as shown by the consistent result from the five pyrosequencing replicates of this extraction, leading to a strong underestimation of the focal species. We excluded these five samples from further analyses (but see Discussion).

Results were strikingly repeatable between replicates from same DNA extractions for both focal species ($R_{Pt.parinus}$=0.920, p<0.001; $R_{Pr.stylifer}$=0.972, p<0.001; Fig. 1). We found a positive linear correlation between the proportion of pyrosequencing reads and the proportion of individuals in the mixture for both focal feather mite species (*Pr.stylifer*: F =418.03, p <0.0001; *Pt.parinus*: F =195.71, p <0.0001). The slope for *Pr. stylifer* was close to one (0.904) and the intercept close to zero (-0.007). The estimated proportion of *Pt. parinus* increased at increasing real proportions of individuals in the samples, with a flatter slope of 0.351 and a higher intercept of 0.307.

Discussion

Our results show that NGS provides a promising tool for quantifying the relative abundance of feather mites from birds. First, we found high consistency among the proportions of individuals of species estimated by NGS from the same DNA extract. This is highly promising because it means that NGS introduced little noise into the data. However, we encountered a potential extraction failure in one of the samples (rightmost points in Fig. 1A), suggesting that the DNA extraction step could bias the abundance estimation by NGS (Kermarrec et al. 2013).

Second, for the two focal species studied here, *Pr. stylifer* and *Pt. parinus*, NGS was very informative in relative terms. That is, the higher the real proportion of a mite species, the higher was the estimated proportion by pyrosequencing. However, the slope of the relationship differed between species. This tells us that, without a previous pilot study, it is not possible to be sure of calculating with precision the real abundance of each species in a sample from the relative frequencies obtained through NGS. Moreover, note that the behaviour of pyrosequencing could potentially change depending on the species composition of the sample (as shown in Fig. 1). The relationship between real and estimated proportions of *Pt. parinus* (Fig. 1) is difficult to explain: when the proportion of *Pt. parinus* was above 50% it was underestimated by pyrosequencing, but it was overestimated below 50%. In other words, the most abundant taxa (either *Pt. parinus* or *Pr. clavatus* / *Pr. sylviae*) was the one being underestimated. Whatever the cause, it seems clear that only in highly controlled scenarios with a known low number of species and conducting pilot studies as the one reported here it could be achieved a reliable estimate of real absolute abundances in a sample. However, pyrosequencing has proved to be a promising tool to compare the abundance of species between samples.

Third, pyrosequencing allowed us to detect individuals present in very low proportions (<6%), supporting previous findings for other groups of organisms (Pochon et al. 2013; Zhan et al. 2013). This is crucial for the detection of elusive species of feather mites expected to be in scarce proportions.

224 Recent literature performing similar studies in other organisms from bacteria to invertebrates
225 have found contradictory results on the power of NGS to calculate the relative abundance of different
226 taxa from a sample (see Introduction). A recent study also questioned the COI-based metabarcoding
227 application, mainly because the primer-binding region is not always highly conserved (Deagle et al.
228 2014). However, as suggested by (Deagle et al. 2014), and as we have found here, COI is an adequate
229 option as a metabarcoding marker in studies where the number of taxa is small and a reference
230 database is available.

231 Overall, although our results are promising, we recommend detailed pilot studies like the one
232 reported here before deciding to apply this method in community studies (see also (Amend et al.
233 2010)), especially when a high accuracy of estimation of the relative abundance of species is needed.
234 However, our study also shows that even when the relationship between the number of reads from
235 NGS and the relative proportion of species is not perfect, the estimated abundance may be corrected by
236 using regression parameters from pilot studies. Overall, we show that at least for analgoid feather
237 mites, metabarcoding through NGS of COI is a highly promising tool to study the evolutionary
238 ecology of the intimate association between birds and feather mites.

239

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248

Compliance with Ethical Standards

Disclosure of potential conflicts of interest

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The authors declare that they have no conflict of interest

Research involving human participants and/or animals

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The study was conducted under the authorization of Junta de Andalucía (permission provided by Consejería de Medio Ambiente), Generalitat de Catalunya (permit number 43430497) and Espacio Natural de Doñana and Dirección General del Medio Natural (permit 2013/27). No endangered species were involved in this study. All birds were studied with non-invasive methods and released at sampling locality few minutes after capture. The protocols were approved by the appropriate ethics committees: the Dirección General del Medio Natural, and the Subcomité de Bioética (CSIC).

Informed consent

Informed consent was obtained from all individual participants included in the study.

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371 **Table 1.** Experimental design showing the number of individual mites in each experimental sample.

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Experimental sample	Feather mite species		
	Proctophyllodes sylviae/clavatus	*Proctophyllodes stylifer*	*Pteronyssoides parinus*
1	1	0	16
2	4	0	16
3	16	0	16
4	64	0	16
5	256	0	16
6	1	16	0
7	4	16	0
8	16	16	0
9	64	16	0
10	256	16	0

373 **Figure captions**

374

375 **Figure 1.** Proportion of reads from NGS vs. real proportion of individuals for *Pteronyssoides parinus*
376 and *Proctophyllodes stylifer* in each experimental sample. The dashed lines represent a line with zero
377 intercept and unit slope, and the continuous lines correspond to the real linear tendency of the points.
378 Crosses correspond to reads of an experimental sample with a problem during DNA extraction (see
379 Results and Discussion).

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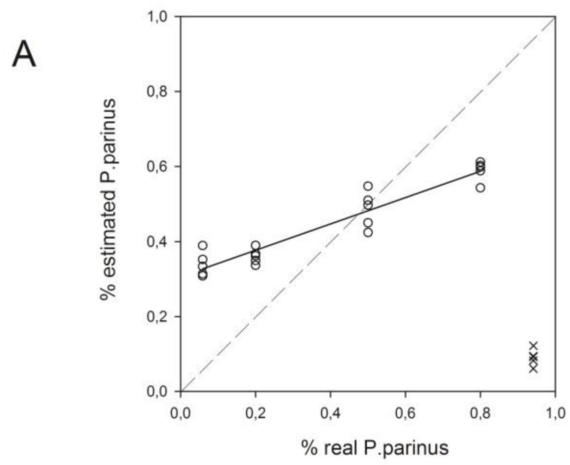

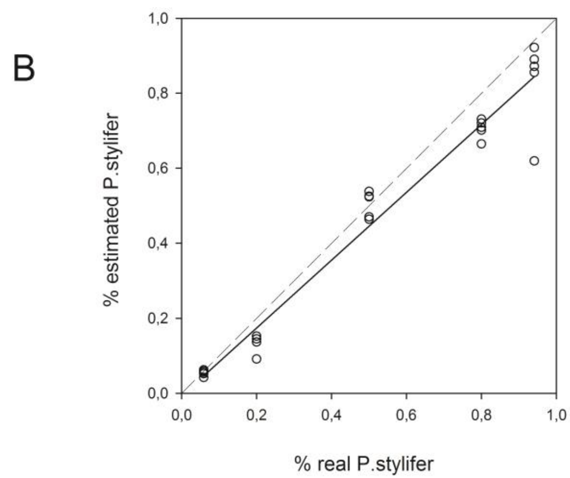

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